# Bridging the gap in translational epilepsy research - A patient brain tissue platform for validating animal data

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Abstract

Drug-resistance is common among patients suffering from epilepsy with about 30 % not responding adequately to the anti-epileptic drugs available today. One of the few alternative treatment options available, is to surgically resect the brain area holding the seizure onset zone.

Despite strong efforts and good preclinical results many new drugs fail during the early stages of clinical testing. For more adequate treatments to be available to the drug-resistant patients the gap between preclinical studies and the clinical trials need to be addressed.

In this thesis, an approach for reducing the gap between the preclinical and clinical research, using resected tissue from epileptic patients is investigated. Optogenetic tools were used in animal models to interrogate seizure mechanisms in an established epileptic neuronal network. For further investigation of seizure mechanisms in patient tissue, a proof-of-concept that optogenetics can be used was demonstrated by successful expression of a light sensitive cat-ion channel, channelrhodopsin. The functionality of the channels were tested during electrophysiological experiments showing strong neuronal depolarisation during light illumination, without compromising the membrane integrity of the cell. Also, a test-platform using resected human brain tissue from drug-resistant epilepsy patients was investigated and further developed. In our platform, acute human hippocampal slices are viable for up to 48 hours with ability to generate epileptiform activity, increasing the time for experiments as well as opening up for gene-therapy approaches to be tested acutely in target tissue. Finally, the anti-seizure effect of Neuropeptide Y, a peptide with seizure-suppressant effect in several animal models, was verified in human tissue using the test-platform, providing support for Neuropeptide Y to be further developed as a treatment strategy for drug-resistant patients.

The work presented in this thesis have demonstrated that resected tissue from epilepsy patients can be used to help close the gap between preclinical and clinical research.The fact, that Neuropeptide Y could reduce epileptiform activity in brain tissue from drug resistant epilepsy patients, validates the platform and provides support for Neuropeptide Y to be further developed as a treatment strategy for patients with drug-resistant epilepsy.



Key words

Epilepsy, Neuropeptide Y, Optogenetics, ChR2, *in vitro*, human, mouse, hippocampus

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*Find your edge*
*Breathe at the edge*
*Long deep breaths*
*On the mat, off the mat*

# Content

# Summary

Epilepsy is a complex but common neurological disease affecting about 50 million people worldwide. It is always characterised by a pathological hyper-synchronised neuronal activity in the brain that manifests as seizures, but the underlying reasons for having epilepsy are many. Due to the complexity of the disease a cure is yet to be found and the treatments available focus on controlling the symptoms, the seizures. Most antiepileptic drugs available today achieve this by decreasing the general activity in the whole brain, which also may have unwanted adverse effects on cognition and memory. Moreover, the antiepileptic drugs available today are ineffective in one third of patients, leaving them with uncontrolled seizures and few alternative treatment options. One alternative treatment option available to some of these patients is to surgically resect the brain area of the seizure onset zone.

The need for new antiepileptic drugs and treatments is evident but despite strong efforts and good preclinical results many new drugs fail during the early stages of clinical testing. For more adequate treatments to be available to the drug-resistant patients, the gap between preclinical studies and clinical trials need to be addressed.

The work in this thesis aim to bridge this gap by using the epileptic tissue, removed from drug-resistant patient during surgical resection to develop a test-platform. The test-platform can be used for testing anti-seizure treatments as well as casting new light on mechanisms behind seizure generation either directly in patient brain tissue, or using this tissue to validate animal data.

First we explored in brain slices from mice the role of inhibitory and excitatory neurons during seizure initiation in an established epileptic network using optogenetics. By electrophysiological measurements, in the subiculum, we show that optogenetic synchronization of inhibitory or excitatory neurons on its own was not sufficient to generate epileptiform activity even in slices from epileptic animals. In contrast to previous studies, we could not trigger epileptiform activity by synchronising interneurons in the presence of the excitability-enhancer 4-Aminopyridine. We could show, however, that

optogenetic synchronization of excitatory neurons in 4-Aminopyridine triggered epileptiform afterdischarges that were driven by GABAergic neurotransmission. To enable further validation of obtained results of seizure generation directly in patient epileptic tissue, we continued by culturing resected human brain slices, and showed a proof-of-concept that optogenetics can be used in human brain tissue.

Also, methods for using resected human brain tissue as a test-platform for new treatments and drugs was investigated and further developed. We demonstrate that acute human hippocampal slices are viable for up to 48 hours with ability to generate epileptiform activity. The increased lifespan of acute human brain slices opens up for more and longer *in vitro* experiments, making it suitable for a viral vector based gene-therapy approach allowing time for transgene expression, and the efficacy to be tested acutely in the target tissue.

Finally, we used the human tissue test-platform to investigate the effects of Neuropeptide Y on epileptiform activity in hippocampal slices from drug-resistant epilepsy patients, as an initial validation step of the test-platform. Neuropeptide Y is a peptide produced in the human brain and released during high neuronal activity. In several animal models this peptide have demonstrated an anti-epileptic and seizure suppressant effect mainly mediated by the Y2 receptor. We have now been able to verify this seizure suppressant effect in hippocampal human brain tissue slices from patients with drug-resistant epilepsy. This finding provides support for Neuropeptide Y to be further developed as a treatment strategy for drug-resistant patients, showing the usefulness of this approach as a translational step for clinical applications of novel treatment strategies that require longer time of acute slice viability.

The work presented in this thesis has demonstrated that resected tissue from epilepsy patients can be used to help closing the gap between preclinical and clinical research.

# Populärvetenskaplig sammanfattning

Epilepsi är en neurologisk sjukdom som drabbar cirka 1 % av alla människor världen över. Sjukdomen kännetecknas av återkommande krampanfall men det finns många olika bakomliggande orsaker till sjukdomen. Anfallen är ett resultat av extensiv aktivitet i en del av eller hela hjärnan och kan se olika ut beroende på var i hjärnan den epileptiska aktiviteten uppstår. Epilepsi behandlas med antiepileptiska läkemedel som minskar risken för anfall genom att sänka aktiviteten generellt i hela hjärnan, något som även påverkar kognition och minnesfunktion. Dessutom fungerar antiepileptiska läkemedel dåligt eller inte alls för en tredje del av patienterna. Ett fåtal av dessa patienter blir hjälpta genom att kirurgiskt ta bort den del av hjärnan där anfallen startar.

Trots att många nya lovande läkemedel har tagits fram med hjälp av preklinisk forskning på djur, är det få av dessa som sedan visar sig fungera i den mänskliga hjärnan och det finns en tydlig klyfta mellan den prekliniska och den kliniska forskningen.

Genom att använda hjärnvävnad som tas bort under kirurgisk behandling av vissa patienter med epilepsi kan man studera den mänskliga epilepsin prekliniskt. Vi har, genom att nyttja den mänskliga vävnaden, försökt minska klyftan mellan den prekliniska forskningen på djur och de kliniska testerna på människor. Vi har utvecklat metoderna kring användandet av vävnaden för att skapa en testplattform där nya läkemedel och behandlingsformer kan testas.

Vi har visat att vävnaden kan hållas vid liv med intakta nervceller kopplade till varandra i sitt nätverk i upp till två dygn efter operationen. I vävnaden har vi lyckats registrera spontan epileptisk aktivitet men också att aktivt framkalla den. Att vävnaden kan hållas i bra skick under lång tid öppnar upp för möjligheten att testa nya behandlingsmetoder som bland annat kan involvera genterapi.

Vi visar att optogenetik, en metod som möjliggör kontroll av specifika grupper av nerver genom aktivering av ljus, kan användas i den mänskliga

hjärnvävnaden. De optogenetiska verktygen möjliggör för framtida studier att specifikt studera hur aktivering av olika nervgrupper påverkar den epileptiska aktiviteten.

Vi använde även vävnaden för att testa en ny behandling som visat tydlig antiepileptisk effekt i prekliniska studier. Behandlingen bygger på en peptid, Neuropeptid Y, som finns naturligt i hjärnan och som bland annat frisätts vid hög aktivitet i avsikt att ha en dämpande effekt på aktiviteten. När vi tillsatte neuropeptiden minskade den epileptiska aktiviteten i den mänskliga hjärnvävnaden och aktiviteten kom sedan tillbaka när peptiden togs bort. Resultatet stämmer väl överens med de tidigare prekliniska studierna och eftersom den mänskliga vävnaden är resistent mot dagens antiepileptiska läkemedel är det sannolikt att peptiden även kan fungera för patienter som inte blir hjälpta idag.

Resultaten i den här avhandlingen visar att mänsklig hjärnvävnad från patienter med epilepsi kan användas för att minska klyftan mellan preklinisk och klinisk forskning för att hjälpa framtagandet av nya antiepileptiska läkemedel.

# Original papers and manuscripts

I. Wickham J, Ledri M, Andersson M, Kokaia M. Optogenetic switch for seizures: Interneurons versus principal neurons in hippocampal network. *Manuscript*

II. Andersson M, Avaliani N, Svensson A, Wickham J, Pinborg LH, Jespersen B, Christiansen SH, Bengzon J, Woldbye DPD, Kokaia M. 2016 Optogenetic control of human neurons in organotypic brain cultures. *Scientific Reports*. 6(1):24818

III. Wickham J, Brödjegård NG, Vighagen R, Pinborg LH, Bengzon J, Woldbye DPD, Kokaia M, Andersson M. 2018. Prolonged life of human acute hippocampal slices from temporal lobe epilepsy surgery. *Scientific Reports*. 8(1):4158

IV. Wickham J, Ledri M, Bengzon J, Pinborg LH, Englund E, Woldbye DPD, Andersson M, Kokaia M. Neuropeptide Y inhibits epileptiform activity in the dentate gyrus of hippocampal tissue resected from drug-resistant epilepsy patients. *Manuscript*

# Abbreviations

| | |
|---|---|
| 0Mg | Excluding magnesium |
| 4AP | 4-Aminopyridine |
| AD | Afterdischarge |
| AED | Antiepileptic drug |
| aCSF | Artificial cerebral spinal fluid |
| AP5 | 2-amino-5-phosphonopentanoic acid (NMDA receptor blocker) |
| CA | Cornu Ammonis (hippocampal region: CA1-CA4) |
| CaMKIIa | Calcium-calmodulin kinase II alpha |
| ChR2 | Channelrhodopsin-2 |
| CS | Cutting solution |
| EEG | Electroencephalogram |
| GABA | Gamma-aminobutyric acid |
| haCSF | Human artificial cerebral spinal fluid |
| hCS | human cutting solution |
| ILAE | International League Against Epilepsy |
| KA | Kainic acid |
| KPBS | Potassium phosphate buffer solution |
| NBQX | $C_{12}H_8N_4O_6S$ (glutamate receptor blocker) |
| NMDA | N-methyl-D-aspartate (glutamate receptor) |
| NPY | Neuropeptide Y |
| PDS | Paroxysmal depolarising shift |
| PFA | Paraformaldehyde |

| | |
|---|---|
| PV | Parvalbumin |
| PTX | Picrotoxin ($GABA_A$ receptor blocker) |
| SE | Status epilepticus |
| SLE | Seizure like event |
| TLE | Temporal lobe epilepsy |

# Introduction

## Epilepsy

Epilepsy is a common neurological disorder affecting about 1 % of the general population, a number corresponding to approximately 50 million people worldwide (WHO 2018). Due to the complexity of the disorder, new definitions describing the disease are generated as the knowledge about it increases. In 2014, a taskforce put together by International League Against Epilepsy (ILAE) published a new practical clinical definition (Fisher et al. 2014a) but the more "general" definition from 2005 still holds true:

> "An epileptic seizure is a transient occurrence of signs and/or symptoms due to abnormal excessive or synchronous neuronal activity in the brain."
>
> "Epilepsy is a disorder of the brain characterized by an enduring predisposition to generate epileptic seizures and by the neurobiological, cognitive, psychological, and social consequences of this condition. The definition of epilepsy requires the occurrence of at least one epileptic seizure."
>
> (Fisher et al. 2005)

It is important to clarify that seizures are the symptoms and manifestations of the disease, epilepsy. The development of epilepsy, epileptogenesis, usually includes several structural and/or chemical changes in the brain. These changes result in a lowered seizure threshold, giving rise to spontaneous seizures or easily triggered seizures. A seizure can be explained as abnormal excessive and/or synchronous neuronal activity in the brain. Depending on where in the brain this abnormal activity is located, the manifestations of this activity will be different. Most of the time a seizure will end by itself after a few minutes, without any intervention, but if the seizure continues it becomes very harmful for the brain and if it continues for more than 30 min a life threatening state called status epilepticus (SE) is reached (Tatum et al. 2001).

## Classification of seizures and epilepsy

Why do some people have a predisposition to generate seizures? There could be many reasons for this. Some are known, such as head trauma, brain tumours, stroke, infection and inherited genetic defects, but in some cases the reason is not known (Duncan et al. 2006, Scheffer et al. 2017). The fact that there are so many different reasons for having seizures shows how complex and multifactorial the disease is. Efforts have been made to classify different types of seizures and epilepsy and an update in classification was published by ILAE in spring 2017 (Scheffer et al. 2017). The new classification is divided into three levels: Seizure type, Epilepsy type and Epilepsy Syndrome, see figure 1.

The idea is to start at the first level, classifying the seizures as either *focal*, *generalised* or *unknown*, then continue to the second level, classifying the type of epilepsy as either *focal*, *generalised*, *combined generalised and focal* or *unknown*. The last level of classification is the epilepsy syndrome, referring to clusters of different features including age-onset, comorbidities, seizure triggers, seizure type, electroencephalogram (EEG) recording and imaging features that tend to occur together.

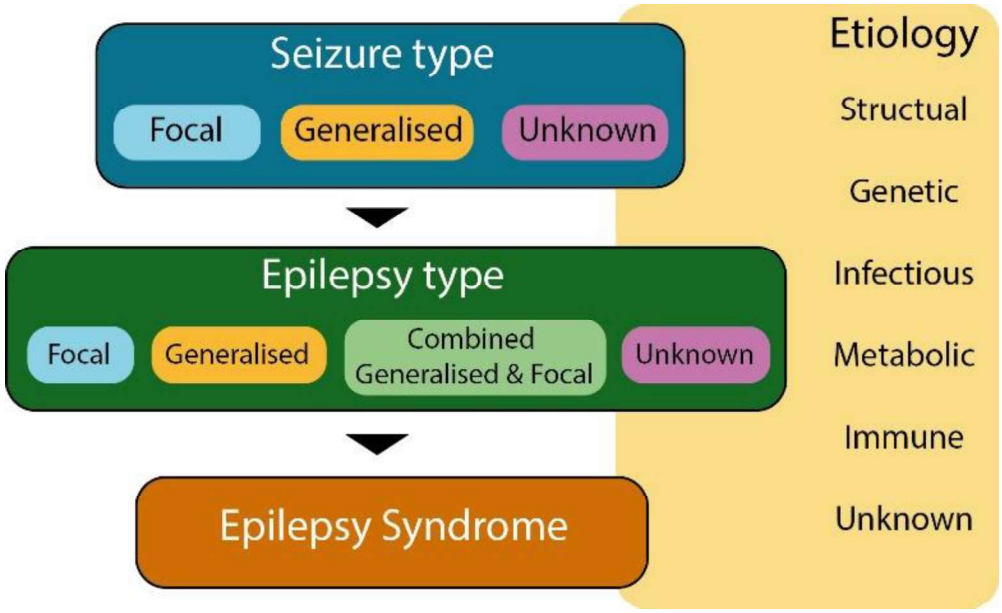


**Figure 1. Classifications of seizure and epilepsy**
Illustation of the new classifications of seizures and epilepsy. Depending on the available techniques a diagnosis can include the seizure type or all the levels from seizure type to epilesy syndrome. At all levels the etiology should be included and more than one etiology can be attributed. *The schematics in the figure is adapted from (Scheffer et al. 2017).*

Seizures are manifested in many different ways and are divided into *generalised* or *focal* when possible. The *generalised seizures* engage both hemispheres from start and a specific focus for the seizure initiation is not located. This type of seizure is usually associated with loss of consciousness and are further divided into "motor" or "nonmotor", depending if the manifestations of the seizure include body movements or not. In opposite to *generalised*, the *focal seizure* start from a specific part of the brain and the patient can be "aware" of what is happening during the seizure (previously called "simple partial seizure"), or "unaware" (previously called "complex partial seizure"). The *focal seizures* are, just as the *generalised seizures*, sub-grouped into "motor" or "nonmotor". *Focal seizures* can also spread to affect the whole brain, they are then further categorised as "focal to bilateral tonic-clonic" (previously named "partial onset with secondary generalisation") (Fisher et al. 2017, Scheffer et al. 2017).

The classification of the type of epilepsy is generally done with the support of EEG recordings. *Generalised epilepsy* is associated with a generalised "spike-wave" (interictal) activity on the EEG and *focal epilepsy* is often accompanied by focal interictal activity. The third category, *combined generalised and focal*, have been added to provide classification of epilepsy types with both seizure types. The *unknown* category in the classification of seizures and epilepsy type can be used when it is clear that the patient have epilepsy but information about the seizures is insufficient to give a classification (Scheffer et al. 2017).

The three different levels of classification enable clinicians to use any of them as a final level for diagnosis, depending on the resources available. The attributed etiology, the underlying cause of the epilepsy, can accompany any of the diagnosis levels. The etiology is divided into the following five groups: *structural*, *genetic*, *infectious*, *metabolic*, *immune* and *unknown*. When applicable, more than one etiology can be chosen. For example if epilepsy is acquired due to a traumatic brain injury the general etiology is classified as *structural* but this category also includes patients with structural malformations with a genetic basis and these patients would, in addition to *structural*, also be classified as a *genetic* etiology (Scheffer et al. 2017).

## Temporal Lobe Epilepsy

The most common form of epilepsy is temporal lobe epilepsy (TLE), characterised by focal seizures originating from structures within the temporal lobe. The seizures have their focus in for example amygdala, temporal neocortex or the hippocampal formation but can also spread and become bilateral tonic-clonic (Engel 2001). In many patients with TLE, the disease is initiated by a traumatic event such as a head injury, infection or febrile seizures and then after a latency period of 5-10 years the first spontaneous seizures appear (O'Dell et al. 2012). TLE is often associated with changes occurring in the hippocampus, including reduction in size, hardening of the tissue and reorganisation with axonal sprouting of the granule neurons in dentate gyrus and neuronal loss, typically most extensive in CA1 and CA3 (O'Dell et al. 2012). Many of the structural changes occurring in hippocampus are referred to as hippocampal sclerosis, scaring of the hippocampus (Blümcke et al. 2013). Epileptogenesis, the development of epilepsy, is believed to take place during the latency period after an initial insult. Some or all of the changes could be subtle at first but will then accumulate as seizures also inflicts an insult to the brain (O'Dell et al. 2012).

## Drug-resistance

About 60 – 70 % of all epilepsy patients are successfully treated with antiepileptic drugs (AEDs) but a large proportion are not helped by today's drugs and continue to have seizures (Duncan et al. 2006). For them, a short list of alternative treatments is left, including ketogenic diet, vagus nerve stimulation and surgical resection. Patients suffering from TLE are overrepresented in the drug-resistant group and as many of them have a focal seizure onset, the resection of the structure holding the seizure onset zone, is sometimes possible. Although only patients with a well-defined seizure focus, in an area reached without compromising important functions such as language and speech, are candidates for surgery.
As seizure freedom is the major determinant for good quality of life for these patients, finding new drugs or alternative treatments that are not only reducing the number of seizures but achieve complete seizure freedom is important (Birbeck et al. 2002).

# Hippocampus

Hippocampus is a neuronal network structure important for memory formation and navigation, with a propensity to develop seizures. The hippocampus, or hippocampal formation as it is formally named, is located deep within the brain in the temporal lobe in each hemisphere. It is divided into distinct regions including dentate gyrus, CA1, CA2, CA3 and CA4 (together referred to as the hippocampus proper), entorhinal cortex and subiculum. The "CA" refer to Cornu Ammonis, "Ammon's horn", named after the mythological Egyptian god Amun Kneph whose symbol was a ram (Amaral & Lavenex 2007). The different regions within the hippocampal formation are linked together by neuronal pathways that relay information mainly going in one direction (Amaral & Lavenex 2007). The major connections are made by the principle cells of the hippocampus, using glutamate as neurotransmitter. They are excitatory neurons named *pyramidal cells* in the CA (due to their pyramidal shape) and *granule cells* (in the dentate gyrus). Sensory and other cortical information is relayed from the entorhinal cortex to the input-area dentate gyrus, continuing via CA3-CA1 and finally reaches the output-area subiculum that connects back to the deep layers of entorhinal cortex, see figure 2. This feed-back loop, creates a seizure sensitive network, believed to be the reason for the big proportion of seizures starting in the temporal lobe. Hippocampus is also largely populated by various types of interneurons that, through the inhibitory neurotransmitter gamma-aminobutyric acid (GABA), can inhibit neuronal output of both excitatory neurons and interneurons. Together with back-propagating excitatory connections, the interneurons make the hippocampus much more interconnected than it may seem at first. The interneurons play a crucial part in the function of hippocampus as they can synchronise activity from several neurons, orchestrating neuronal firing into oscillatory activity.

The synchronous network activity in the hippocampus is important for normal functions and the activity is divided into three oscillation levels (reviewed in Colgin 2016). The level with the lowest frequency range, called Theta (4-12 Hz), occurs during explorative behaviour, locomotor behaviours and rapid eye movement during sleep (Vanderwolf 1969). Gamma (25-100Hz) can be separated into slow and fast gamma oscillations with the fast rhythm associated with memory encoding (Jutras et al. 2009, Sederberg et al. 2007). The last range, called Sharp-Wave ripple complex (110–250 Hz ripples superimposed

on 0.01–3 Hz sharp waves) is seen during sleep and is believed to have an active part in memory consolidation (Axmacher et al. 2008).

The experimental work in this thesis is mainly focused on two areas within hippocampus, namely dentate gyrus and subiculum. These two areas are important input (dentate gyrus) and output (subiculum) structures with the potential ability to "gate" or stop seizures entering into the hippocampus and to "contain" or stop seizures from propagating out of the hippocampus. They are also two of the three areas (the third is CA2) that generally are spared during hippocampal sclerosis, with less neuronal death.

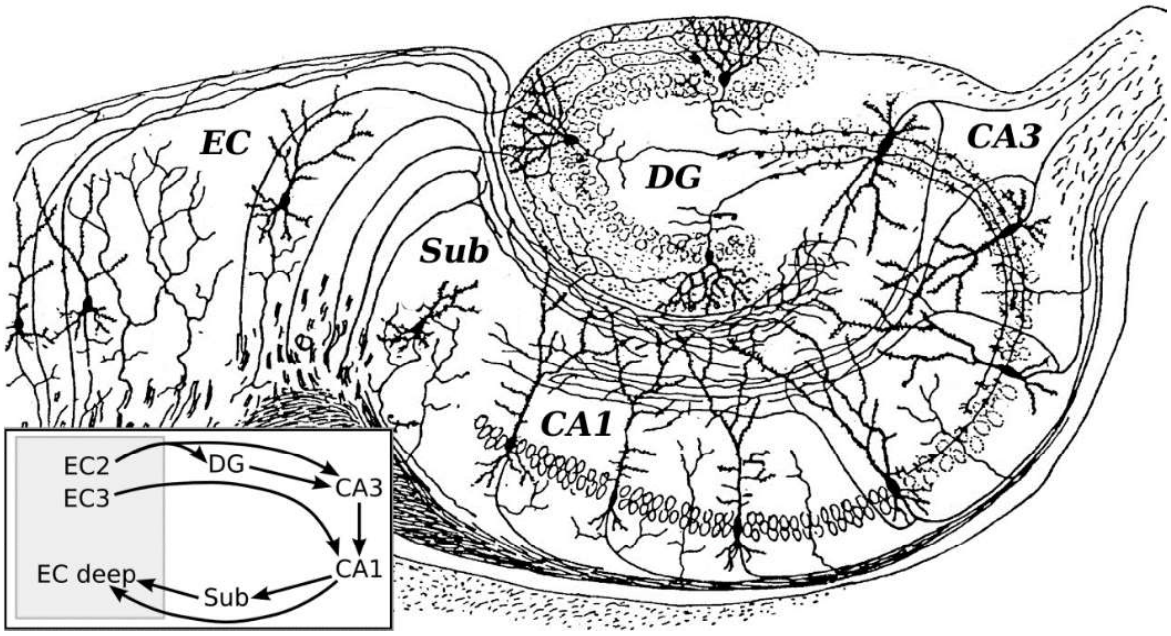


**Figure 2. Neuronal network organisation in Hippocampus**
Dentate gyrus, DG, recives information from entorhinal cortex, EC, and relays information forward to the CA3 and finally CA1. From CA1 the information connects back to the deep layers of entorhinal cortex via subiculum, Sub. The granule cells in the dentate gyrus and pyramidal cells are exemplified along the whole structure with the network connections beween each area.
*Image adapted from the work of Santiago Ramón y Cajal (1911), © Public Domain, Wiki Commons: https://commons.wikimedia.org/wiki/File:CajalHippocampus_(modified).png*

# Resected human tissue

When a resection of an epileptic zone is performed it provides a unique opportunity. Not many researchers within the field of neurology are fortunate to work with the target tissue in an *in vitro* set up. The first work investigating epilepsy in an *in vitro* setup with resected tissue from epilepsy patients, was published by Schwartzkroin and Prince in the mid-1970s. Schwartzkroin was also the first to report spontaneous epileptiform activity in human brain slices (Schwartzkroin & Haglund 1986). Since then, more have followed and in the late 1990s the first systematic search for spontaneous epileptiform activity was conducted resulting in field recordings of "sharp waves" discovered in temporal neocortical slices resected from epilepsy patients (Köhling et al. 1998). Both ictal (Gabriel et al. 2004, Huberfeld et al. 2011, Remy et al. 2003) and interictal (Cohen et al. 2002, Huberfeld et al. 2007, Wozny et al. 2003, 2005) activity (see explanation under the headline: Epileptiform activity) have been studied in hippocampal slices. As well as spontaneous epileptiform activity generated within subiculum (Cohen et al. 2002, Huberfeld et al. 2007) and CA2 (Wittner et al. 2009).

The studies from human hippocampal tissue help to gain more knowledge about the mechanisms of epileptic activity but can also be used to bridge the gap between animal research and the clinic (Ledri et al. 2015).

Hippocampal tissue resected from patients with epilepsy is not only target tissue because it is human, but also because it has been shown to be drug resistant (Jandová et al. 2006, Remy et al. 2003, Sandow et al. 2015). The tissue provides us with the possibility to test novel treatments and drugs that have been developed and studied in animal models, before continuing to costly clinical studies (Klaft et al. 2016).

The tissue can be used acutely (after slicing and 3 hours rest) but also after longer incubation (several weeks) in a controlled environment, as organotypic slices. Acute slices are generally kept and recorded in an interface chamber with nutrients and ions in a solution mimicking the human cerebral spinal fluid under the slice, in a steady flow, and humidified air above, to maximise the oxygen supply. The amount of oxygen available for the slices is important when studying epilepsy and seizures, as the neurons need oxygen to be able to generate action potentials at a high frequency (Cohen et al. 2002, Cunningham et al. 2012, Jones et al. 2016).

It is however important to remember that the tissue has been traumatised during the resection (cutting of blood supply and compressed when removed) and then sliced into thin slices. This *in vitro* experimental setting with a reduced neuronal network and no input from other brain structures limits the questions to be explored and cannot replace animal studies or clinical trials. But for the same reasons the controlled *in vitro* setting also gives the opportunity to study the effects and mechanisms of new drugs as well as the mechanisms behind seizure initiation and termination.

# Epileptiform activity

Spontaneous seizures arise from hyperexcitable and hypersynchronous neuronal networks in the brain. How do we know it is a seizure when the activity is recorded from a brain slice? With only a minor part of the brain it might not be obvious what is normal activity and what is epileptic activity. By comparing activity recorded in slices, to what has been recorded *in vivo*, that is, in patients or in animal models, the activity recorded from a slice can be verified as epileptic (Fisher et al. 2014b).

## Definitions

There are many ways to describe the abnormal, presumably epileptic activity recorded from slices. It can sometimes be confusing when the words are not accompanied by a definition. This section is an attempt to define and clarify the meaning of the words used in this thesis. A typical hallmark for epileptic activity in a neuron is the paroxysmal depolarising shift (PDS). The PDS is a fast shift in membrane potential in the positive direction (depolarising), with or without action potentials, followed shortly by a shift back down to "normal" membrane potential again, see Figure 3C. In an EEG recording the PDS manifests as an interictal sharp wave or spike.

Ictal and interictal are words originally used in the clinic to describe two different type or parts of epileptic activity seen in an EEG recording. They are used when describing a seizure (ictal) and the activity seen between seizures (interictal). With this definition, the interpretation is that ictal activity is the abnormal brain activity during a seizure. In a focal seizures it usually starts as a fast, low amplitude activity with desynchronising background activity, which

can last for tenths of seconds. The activity then evolves into irregular spiking patterns (the "tonic" phase), then synchronous discharges (the "clonic" phase) that usually terminates the seizure with synchronous periodic bursting (after discharges). The interictal activity is abnormal brain activity (usually spikes or sharp waves on an EEG recording) occurring in-between seizures (Fisher et al. 2014b). For *in vivo* recordings made in animal epilepsy models, there is no consensus on an exact definition but generally interictal activity is ascribed short epileptic discharges and ictal activity have been described to last longer than 2-3 seconds (Fisher et al. 2014b), see figure 3A and B. To discriminate between ictal and interictal by only looking at duration is however problematic and could be misleading (Fisher et al. 2014b). These words, ictal and interictal, are also used to describe similar activity seen in electrical recordings from slices.

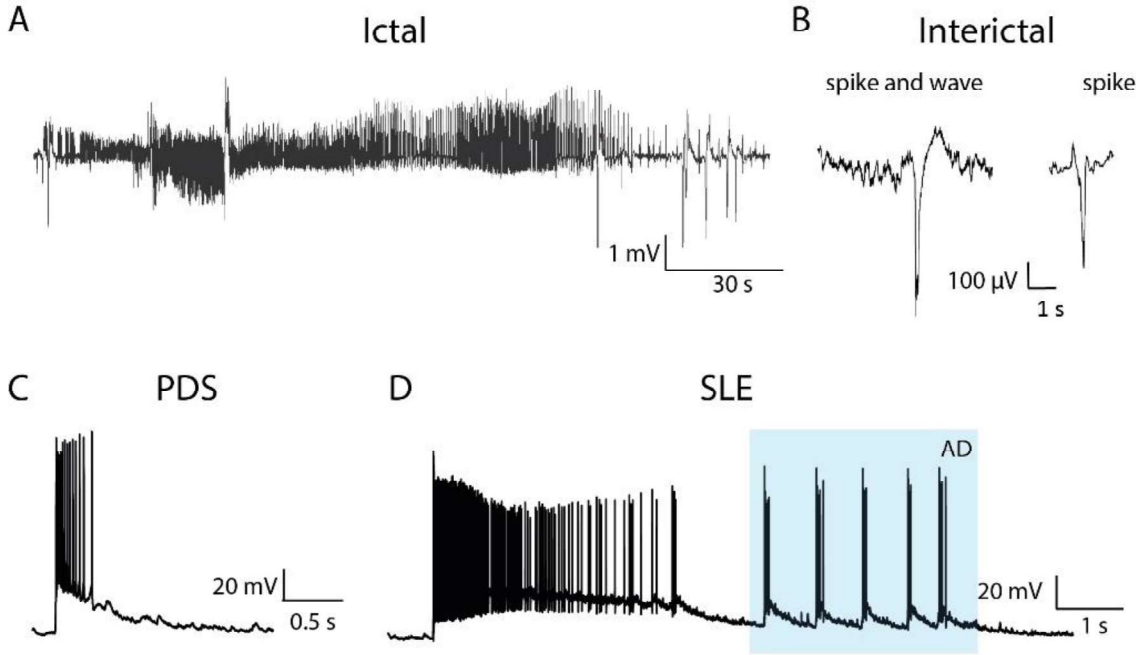


**Figure 3. Example of different epileptiform activity.**
Ictal (A) and interictal (B) activity from *in vivo* intrahippocampal recordings in rodent. PDS (C), SLE (D) and AD (blue square) recorded from resected human tissue slices. *Panel B is modified from Chauvière et al. 2012.*

Other ways to describe the abnormal, and presumably epileptic activity recorded in slices, is seizure like event (SLE). A SLE starts with very high frequent activity (action potentials) superimposed on a paroxysmal depolarisation that continues with less frequent activity (action potentials), and

ends with terminating bursts (Fisher & Scharfman 2014), see figure 3D. The start of the SLE could be seen as ictal-like and the terminating bursts as afterdischarge (AD). The ADs are often seen after strong synchronous stimulation (electric or optogenetic) that is thought to mimic ictal activity (in *paper I* they are called: stimulation-induced ictal epileptiform AD) (Berglind et al. 2018, Ellender et al. 2014, Sessolo et al. 2015, Stasheff et al. 1985).

## In vitro models of epilepsy

To generate epileptiform activity in brain slices, several alterations to the ionic composition of the perfusion medium can be made to create a hyperexcitable environment. The ionic modifications itself, or in combination with electrical stimulations or specific receptor or channel blockers, will trigger epileptiform activity (Avoli & Jefferys 2016). During the work with this thesis epileptiform activity was generated by adding a potassium channel blocker and/or by excluding magnesium from the perfusion medium.

*4-Aminopyridine*

Application of the potassium channel blocker 4-Aminopyridine (4AP) is a widely used method to generate robust epileptiform activity in rodent and human hippocampal slices. The activity resembles the interictal activity seen in EEG recordings from epilepsy patients (Hsiao et al. 2015, Jones et al. 2016, Traub et al. 1995). It acts by selectively blocking voltage-gated potassium channels of the $K_v$-1 family, leading to a broadening of action potentials that results in increased neurotransmitter release (Avoli et al. 2002, Buckle & Haas 1982, Rutecki et al. 1987). 4AP affect both excitatory and inhibitory neurons, thus both the release of glutamate and GABA will increase. At first, a fast interictal activity in the form of PDS appears in the CA3 and then later also a slower and longer interictal activity, characterised by long-lasting depolarisation with a few action potentials. A fixed origin have not been found for the slower interictal activity and even with the same concentration of 4AP, the slow interictal activity can differ in shape, occurrence and rate (Avoli & de Curtis 2011). Ictal-like activity, with long durations (>30s), an initial high frequency component and terminating ADs, resembling ictal events recorded in vivo, have also been reported (Avoli & de Curtis 2011).

*Excluding magnesium*

Normally, magnesium blocks the glutamatergic NMDA receptor until a depolarisation of the membrane will force the magnesium ion out and enable passage of positively charged cations to flow from the outside of the cell to the inside, making the depolarisation even stronger. This function acts as a coincidence detector (Gustafsson et al. 1987), an important part of our memory function (Morris 2013). By excluding magnesium (0Mg) from the perfusion media the magnesium block is removed and NMDA-channel will have a higher probability to be open, resulting in the propensity to generate more action potentials. The epileptiform activity is similar to what has been seen during 4AP application, ictal events resembling *in vivo* recordings, as well as fast and slow interictal activity (Avoli et al. 2002, Köhling et al. 2000).

## Animal models of epilepsy

To study the symptoms and underlying mechanism of epilepsy several different animal models are used. The two main models used in rodents are electrically (kindling) and chemically induced epilepsy. In *paper I* we use the kainic acid (KA) model (chemically induced).

*Kainic acid model*

The KA status epilepticus model (reviewed in Lévesque & Avoli 2013) is generally used as a model of TLE because of its resemblance to the human condition, with an initial trauma (status epilepticus), a latent period and then spontaneous recurrent seizures (Ben-Ari 1985, Victor Nadler 1981). The initial trauma is induced by systemic injection of KA resulting in status epilepticus. The KA is an glutamate analogue that will induce robust neuronal depolarisation and eventually cell death (Bloss & Hunter 2010, Vincent & Mulle 2009) by binding to glutamate receptors of KA subtype. Within the central nervous system, KA receptors are predominantly found in the hippocampus and they are located both pre- and postsynaptically (Bloss & Hunter 2010). After the initial insult, several changes associated with TLE will take place, including hippocampal sclerosis, mossy fibre sprouting and cell death (Davenport et al. 1990, Lévesque & Avoli 2013). During the latent period, two types of interictal activity have been observed. One is characterised by a spike followed by a long wave and the other is only a spike with no wave (figure 2B) (Chauvière et al. 2012). Non-convulsive seizures occur 10-30 days

after systemic injection of KA and a clustering of interictal activity is seen in rats that later develop epilepsy (White et al. 2010).

# Optogenetics

Optogenetics is a method where, by introducing light sensitive ion-channels to neurons via genetic manipulation, it is possible to control the neuronal activity by using light. This method, emerging from the discovery of light-activated ion channels and pumps (Nagel et al. 2002, 2003; Zhang et al. 2007b), used in mammalian cells for the first time by Karl Deisseroth and Ed Boyden in 2005, is very useful due to the ability to target specific neuronal populations (determined by the promoter) and its high temporal resolution. Given the method's enormous advantages, it is now used in neuroscience-laboratories all over the world (Adamantidis et al. 2015).

Channelrhodopsin-2 (ChR2) was the first light activated channel to be developed and proven functional in mammalian hippocampal neurons (Boyden et al. 2005). It is a cat-ion channel that will open when illuminated with blue light, letting small positively charged ions such as $Na^+$ to pass over the membrane and into the cell. The activation of ChR2 will depolarise the cell and within milliseconds trigger one or several action potentials. Several light sensitive channels and pumps have been developed and it is now possible to both excite and inhibit population of cells by using light of various wavelengths (Lin 2011, Zhang et al. 2007b).

To target a specific cell population, the sequence of the rhodopsin can be combined with a promoter specific for the cell population of interest (Zhang et al. 2007a). The expression can also be limited to a certain brain area by careful injection of viral vector in to the specific area. Several transgenic mouse-lines expressing different rhodopsins under various promoters have been developed (Zeng & Madisen 2012).

Within the epilepsy research field, the optogenetic tools have not only been used to study mechanisms behind seizures and epileptogenesis but was early acknowledged as a potential future treatment option. Several studies have provided proof of principle for an optogenetic seizure suppressant treatment both *in vitro* (Tønnesen et al. 2009) and *in vivo* (Berglind et al. 2014, Krook-Magnuson et al. 2013, Paz et al. 2013, Sukhotinsky et al. 2013, Wykes et al. 2013).

# Neuropeptide Y

Neuropeptide Y (NPY) is an important peptide that acts as a neurotransmitter or neuromodulator, with many different functions in several brain areas. It is an endogenous peptide that is produced and released by interneurons throughout the mammalian brain (Hendry et al. 1984).

## NPY and its receptors

NPY consists of 36 amino acids (Tatemoto et al. 1982) and regulates a broad variety of functions such as anxiety, pain, food intake, depression, drug addiction and excessive hyper excitability (Benarroch 2009).

So far, five different NPY receptors have been detected and cloned (Y1, Y2, Y4, Y5 and Y6) (Michel et al. 1998, Silva et al. 2005). All of the receptors are within the family of G protein-coupled receptors but only the Y1, Y2 and Y5 have an active role in the mammalian brain. Y4 have a very small affinity for NPY and Y6 is only present and functional in some mammals and is not functional in humans (Lundell et al. 1995, Starbäck et al. 2000). The different receptors elicit different responses when activated and the abundance of each receptor varies with different brain regions and also to some extend between different mammal species (Michel et al. 1998, Redrobe et al. 1999, Silva et al. 2005). The Y1 receptor is mostly found in the neocortex but is not as abundant in the hippocampus (Dumont et al. 1998). Activation of the Y1 receptor has been shown to counteract inflammatory processes and to reduce the number of activated microglia (Ferreira et al. 2010, 2012). Y1 receptor is also mediating the constriction of blood vessels within the cortex (Uhlirova et al. 2016). The Y5 receptor has been shown to stimulate autophagy in the hypothalamus, both *in vitro* and *in vivo* (Aveleira et al. 2015). Both Y1 and Y5 receptors are located postsynaptic, on the receiving end of the synapse. Only the Y2 receptor is located presynaptically, on the transmitter releasing end of the synapse (Stanić et al. 2011). The Y2 receptor is the most abundant NPY receptor in the human brain, with the highest expression found in dentate gyrus (Caberlotto et al. 1998). Both NPY and its Y2 receptor is upregulated (increased) in the hippocampus after a seizure, both in rodents and humans (Furtinger et al. 2001, Mathern et al. 1995). NPY is therefore believed to be an endogenous anticonvulsant, a way for the neurons to counteract the extensive action

potential firing (Furtinger et al. 2001, Schwarzer et al. 1996, Vezzani et al. 1996).

### NPY as seizure suppressant

NPY has been shown to have a seizure suppressant effect in many animal studies, *in vitro* and *in vivo* (Bijak 1999, Chiu et al. 2018, Deborah Lin et al. 2006, El Bahh et al. 2005, Foti et al. 2007, Klapstein & Colmers 1997, Noe et al. 2010, Noè et al. 2008, Richichi et al. 2004, Sørensen et al. 2009, Woldbye et al. 2002) and the Y2 receptor has been identified as the mediator of this effect in rodents (El Bahh et al. 2005, Nikitidou Ledri et al. 2016, Vezzani et al. 2002, Woldbye et al. 2010). So what happens when the NPY binds to an Y2 receptor? When the Y2 receptor is activated by NPY it initiates a cascade of events within the synapse resulting in a lower probability for release of neurotransmitters. This inhibitory effect is due to a de-activation of the voltage gated $Ca^{2+}$-channels controlling the release of neurotransmitters (Colmers & Bleakman 1994, Qian et al. 1997).

As NPY expression have been preserved in mammals throughout evolution, the seizure suppressant effect observed in rodents is likely translational to humans (Larhammar 1996). In our lab, the effect of NPY in human brain tissue has previously been studied in 2015 by Marco Ledri, who showed that NPY supressed excitatory synaptic transmission in the dentate gyrus of hippocampal slices. The effect was abolished when a Y2 receptor antagonist was applied, suggesting that the NPY was acting through the Y2 receptors (Ledri et al. 2015).

## From animal research to the clinic

The discovery of over 15 new AED, during the last three decades have been possible through preclinical animal research, providing patients and physicians with more options for the treatment of epilepsy. However, even the new AEDs fail to control seizures in about 30% of the patients (Duncan et al. 2006, Löscher et al. 2013). The present AEDs only act on the symptoms, the seizures, by dampening excitability in the whole brain, leading to reduced ability for learning and impaired cognition (Perucca & Gilliam 2012). They cannot prevent epilepsy from developing or treat the epilepsy (Piccenna et al. 2017,

Temkin 2009). Furthermore, very few clinical trials aiming to treat epilepsy have been initiated since year 2000 (Klein & Tyrlikova 2016). In the year 2000 the goal, set by National Institute of Health (in United States of America), was to treat epilepsy but this has changed with time into aiming at preclinical research with no mentioning of clinical trials (Klein & Tyrlikova 2016). Partly the reason for this is due to the fact that the few drugs entering clinical trials have failed, leaving the pharmaceutical companies with huge investments and no new drug, making it even harder to justify new clinical trials (Simonato et al. 2016).

Why is it so hard to translate animal research to the clinic? This is not only a problem within the epilepsy research field, but is seen in general with 85% of early clinical trials failing despite successful preclinical testing (Mak et al. 2014). Problems with study design, lack of multi-centre preclinical studies and focusing too hard on only one animal model are often believed to be the reason for failure (Perrin 2014). But there are additional challenges for epilepsy as the mechanisms for the disease is not clearly understood.

Efforts are made to turn this trend around and find ways to improve the ability to predict efficacy, tolerability and to aim for the unmet needs of the patients that are not responding to today's drugs. A review by Löscher from 2011, covering the most common animal models for epilepsy and seizures, highlight the importance of choosing the right model depending on the main purpose of the study. For example an acute seizure model is relatively simple and easy to use, but not an appropriate model for testing new antiepileptogenic treatments, as the changes associated with epileptogenesis are not included in acute seizure models (Löscher 2011). Multi-centre preclinical studies modelled on phase 2 and 3 clinical trials have been suggested to aid in closing the gap between animal studies and the clinic (O'Brien et al. 2013, Simonato et al. 2016).

In order to find new drugs and treatments, that can give seizure freedom to patients resistant to today's AEDs, it is important to use models that are drug resistant, for example post-SE TLE models (Löscher 2011) and resected human hippocampal slices (Jandová et al. 2006, Remy et al. 2003, Sandow et al. 2015). New possibilities are now also opening with gene therapy, for example to give a more local treatment, which will not affect the whole brain but only the seizure focus, possibly reducing the adverse effects seen with today's drugs.

# Aims of the thesis

The overall aim of this thesis was to validate experimental data from animal models in human epileptic brain tissue, with the specific focus on the role of different neuronal populations in seizure initiation, as well as develop and use this platform in validating novel anti-seizure treatment strategies.

The work was divided into four separate parts, each with a specific aim:

I. Investigate, in rodent brain slices, if synchronising different populations of neurons, with optogenetics, can trigger epileptiform activity in an established epileptic network *(paper I)*

II. Show proof-of-concept that optogenetic tools can be expressed in resected epileptic human tissue slice cultures *(paper II)*

III. Develop a test-platform where acute human tissue slices can be kept longer, enabling more time for experimental work and viral vector expression *(paper III)*

IV. Provide an initial validation of the acute human tissue slice platform in evaluating therapeutic strategies for seizure-suppression using NPY *(paper IV)*

# Experimental procedure

## Animals

All animals were bred and kept in the local animal facilities in standard cages on a 12 hour light/dark cycle with free access to food and water. All experimental procedures were approved by the local (Lund/Malmö) Animal Research Ethics Board (Permit M47-15 and M49-15). Animals were used for the experimental work in *paper I.*

### Transgenic mice

The work in *paper I* involved two different transgenic mouse lines: PV::ChR2 and CaMKIIa::ChR2. These transgenic mouse lines were produced by mating Ai32(RCL- ChR2(H134R)/EYFP) (Jackson #012569) mice with either PV-Cre (Jackson #008069) or CaMKIIa-Cre (Jackson #005359) respectively. The PV::ChR2 mice express ChR2 in PV-interneurons enable activation of this specific type of interneuron when exposed to blue light. The CaMKIIa promoter targets principle neurons (excitatory) with a strong expression in hippocampus (Wang et al. 2013) enabling activation when illuminated with blue light.

### Status Epilepticus

SE was induced to generate mice with spontaneous recurrent seizures, providing a hippocampus that had undergone the structural changes associated with epilepsy. To induce SE, freshly prepared KA was injected intraperitoneally, in low doses, in a titration fashion to enhance the survival rate of the injected mice (4-6 weeks of age) (Puttachary et al. 2015, Tse et al. 2014). The mice were monitored continuously until grade 5 seizures or SE was reached with seizures rated according to the Racine scale (Racine 1972). Three

to five weeks after SE the mice were decapitated and hippocampal brain slices were used in electrophysiological experiments.

## Human Tissue

Prior to the hippocampal or cortical resection, the patients undergo an extensive pre-surgical workup to evaluate if the benefit of removing the tissue, outweigh the risks of performing the surgery. The pre-surgical investigations included neurological and neuropsychological assessment, extracranial video-EEG recording and neuroimaging using magnetic resonance imaging. If needed, invasive EEG monitoring using intracranial electrodes (subdural strip or grid electrodes with or without intracerebral depth electrodes) was performed. Written informed consent to use tissue for research purposes was obtained from all patients prior to surgery. The use of resected patient tissue and procedures were approved by the local Ethical Committee in Lund (#212/2007) and Copenhagen (H-2-2011-104) and were performed in accordance with the Declaration of Helsinki.

During the hippocampal resection, the surgical team removes a piece of the skull to expose part of the temporal lobe. The lateral neocortex of the anterior middle- and inferior gyrus was removed in the process of reaching the hippocampal formation, positioned adjacent to the temporal horn of the lateral ventricle deep within the brain. Blood vessels were carefully coagulated around the tissue to be resected to prevent bleeding. The hippocampus was removed *en block* (in one piece) and then cut in three pieces, the anterior one third as well as the posterior one third of the resected tissue was used for pathological evaluation and the middle one third for electrophysiological experiments. The piece was directly submerged in to ice-cold human cutting solution (hCS) containing (in mM): 200 sucrose, 21 $NaHCO_3$, 10 glucose, 3 KCl, 1.25 $NaH_2PO_4$, 1.6 $CaCl_2$, 2 $MgCl_2$, 2 $MgSO_4$ (pH 7.4, osmolarity 300–310 mOsm), transported to the laboratory. Resected tissue from Lund University Hospital was transported, by foot, to the adjacent building. For resections performed in Copenhagen University Hospital, the tissue was transported by car, for about one hour, traveling in a closed container with ice-cold hCS.

# Slice preparation

All slicing was performed with a Vibratome (VT1200S, Leica Microsystems) and a cooling chamber connected to a minichiller (Huber). The razor blade of the Vibratome vibrates in the horizontal direction to ensure a less traumatic sectioning of the tissue. The vibration amplitude was set to 1.7 mm and the forward speed through the tissue was 0.05 mm/s. The tissue was cut into 400 µm (*paper I, III* and *IV*) or 250 µm (*paper II*) thick slices.

## Mice brain slices (*paper I*)

The mice were lightly sedated with isoflurane and decapitated with a pair of scissors. The head was directly submerged into ice-cold cutting solution (CS) containing (in mM): sucrose 75, NaCl 67, $NaHCO_3$ 26, glucose 25, KCl 2.5, $NaH_2PO_4$ 1.25, $CaCl_2$ 0.5, and $MgCl_2$ 7 (pH 7.4, osmolarity 305–310 mOsm), continuously oxygenated. The scull was dissected from neck to nose, exposing the brain by carefully removing the bone with a pair of tweezers. The hemispheres were then separated with a scalpel and the cerebellum was detached and discarded. Each hemisphere was lifted out of the cranium with a spatula and the "magic cut" (Bischofberger et al. 2006) was performed across the dorsal hemisphere to provide a surface on which the hemisphere was glued on to the slicing platform. This platform was placed in the slicing chamber and the tissue was fully submerged in CS continuously oxygenated and kept at 3˚C. As horizontal slices were cut from the hemispheres, they were transferred to a pre-incubation chamber with artificial cerebral spinal fluid (aCSF) containing (in mM): NaCl 119, $NaHCO_3$ 26, glucose 11, KCl 2.5, $NaH_2PO_4$ 1.25, $CaCl_2$ 2, and $MgSO_4$ 1.3 (pH 7.4, osmolarity 295–305 mOsm), heated to 32˚C and continuously oxygenated. In this chamber the slices were incubated fully submerged and resting on thin nets 15-30 min before they were moved to lens-paper cuttings, resting on a net placed on the aCSF surface in a beaker. The beaker is placed in a closed Plexiglas box with continuous oxygenation of the aCSF, creating an interface resting chamber with humidified air above the slice and aCSF beneath. The slices rested for at least 1 hour before they were individually moved to the electrophysiological setup.

## Human brain slices (*paper II-IV*)

Upon arrival in the laboratory the orientation of the tissue was established and sometimes trimmed with a scalpel to provide a better slicing direction. The tissue was then glued onto the platform and placed in the vibratome cooling chamber, completely submerged in continuously oxygenated ice-cold hSC, see figure 4A.

For the experiments with organotypic cultures, *paper II*, the tissue was cut into 250 µm thick slices and moved to ice-cold rinsing medium containing Hank's balanced saline solution with HEPES 20 mM, glucose 17.5 mM and 0.5% penicillin/streptomycin. They were then moved to the cell culture laboratory where they were transferred to cell culture inserts (Millipore) resting in medium contained 50% minimum essential media, 25% horse serum, 18% Hank's balanced saline solution, 2% B27 supplemented with 0.5% penicillin/streptomycin solution, glutamine 2 mM, glucose 11.8 mM, and sucrose 20 mM placed in six-well plates in an incubator holding a 90% humid environment at 37˚C, 5%$CO_2$ and 95% $O_2$. After 12 hours the viral vector (LV-Syn-hChR2(H134R)-EYFP) was applied as drops onto the slices and the slices were then incubated for 2 weeks with fresh medium added three times a week. B27 was removed from the medium after the first week of culturing.

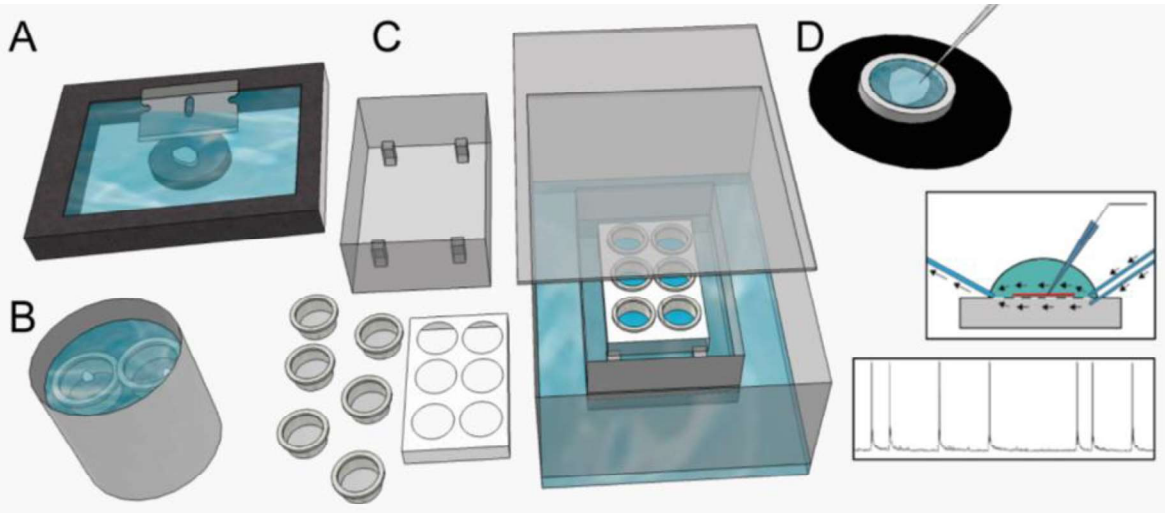


**Figure 4. Acute incubation setup for human tissue.**
The tissue was first cut into thin slices (A) and then moved to the pre-incubation bath (B). The slices were then transferred to cell culture inserts and placed in the interface incubation (C). For electrophysiological recordings the slices were individually moved to a dual-flow recording chamber with a flow of perfusion medium above and below the slices to maximise the anmount of oxygen available (D).

In experiments with acute incubation, *paper III* and *IV*, the tissue was cut into 400 µm thick slices, which were individually transferred to a pre-incubation chamber where they rested fully submerged in 34˚C human aCSF (haCSF) containing (in mM): 129 NaCl, 21 $NaHCO_3$, 10 glucose, 3 KCl, 1.25 $NaH_2PO_4$, 2 $MgSO_4$, and 1.6 $CaCl_2$ (pH 7.4, osmolarity 300–310 mOsm), continuously oxygenated, for 15-30 min before they were moved to the interface incubation chamber. In the interface incubation chamber the slices rested on cell culture inserts placed on the surface of a continuous flow of haCSF. The procedure is illustrated in figure 4 and further explained in *paper III*.

# Electrophysiology

The results in this thesis relay mostly on electrical recordings from individual neurons. Whole-cell patch-clamp recordings can give information about a cell's wellbeing and synaptic events by interpreting the electric physical quantities such as current, potential and resistance. To perform a successful whole-cell recording the famous whole-cell patch-clamp technique was practiced, see figure 5C.

## Principles of whole cell recording

By using a gentle suction as the tip of a glass recording pipette is pressed against the cells membrane a tight seal is formed. This seal is called the "giga-Ohm seal" because the electrical resistance is reaching giga-ohm range. The cell is now securely attached to the recording pipette and a sharp suction will rapture the part of the membrane that is encircled, while leaving the seal intact, see figure 5C. This opening allows the recording electrode full access to the cells cytoplasm and the electrical properties and activities can now be detected, amplified and recorded. It is important to remember that by gaining access to the cells interior the solution in the pipette will start to diffuse into the cell and inevitably affect its properties. The composition of the pipette solution should be chosen with care and can differ depending on the purpose of the recording. The whole-cell recording can be done in two different modes, voltage clamp or current clamp. In voltage clamp the potential is controlled by the experimenter and usually set to -70 mV while the current through the pipette (in the range of pA) is recorded. Using current clamp mode, the current is controlled and the membrane potential is recorded. By using these different

modes it is possible to measure the cells resting membrane potential, action potential threshold and firing pattern as well as synaptic events. Through the intracellular (the pipette) and the extracellular (the bath) solutions is it possible to manipulate the environment. The addition of for example synaptic agonists or antagonists to the bath solution can aid in discriminating between different types of synaptic transmission or be used to enhance the probability for action potentials and thereby pushing the neural network to generate epileptiform activity.

## Principles of field recording

Field recordings are performed by placing a recording pipette in the specific area of interest. The recording pipette is not attached to a specific cell but lies in the extracellular environment and will not be able to pick up on single cell activity, see figure 5A. Although if several cells are active at the same time this will create a stronger electrical change, a field potential, which will be detected. This recording technique may seem blunt compared to whole-cell patch-clamp recording but is very important as it can tell if several cells are engaged in a specific activity.

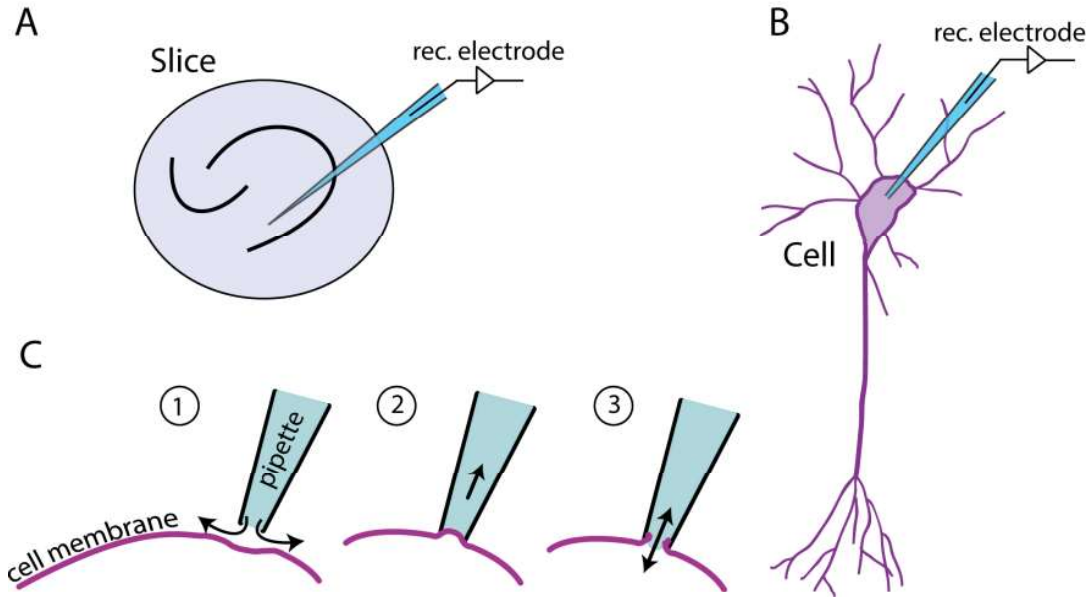


**Figure 5: Field and whole-cell patch-clamp recording.**
In a filed recording (A) the pipette is placed in the extracellular space, recording activity from several cells. Whole-cell recordings (B) are successfully performed using the patch-clamp technique (C) where the pipette form a tight seal with the cell mebrane (1, 2) and the membrane under the pipette is ruptured (3), leaving the seal intact.

## Setup

For all electrophysiological experiments an EPC-10 amplifier (HEKA Electronik) was used together with the Patchmaster software, acquiring data with a sampling frequency of 10 KHz. Electrodes were made from borosilicate glass pipettes pulled with a Flaming-Brown horizontal puller (P-97, Sutter Instruments) and backfilled with pipette solution (whole-cell patch-clamp recording) or aCSF (field recording), with a silver electrode placed inside. All whole-cell patch-clamp recordings were performed with a pipette resistance between 3 and 6 MΩ. Pipettes for field recordings had a resistance of 1-3 MΩ. For visual guidance, a differential interference microscope (Olympus) with a 10x or 20x (submerged) objective was used. The microscope and electrodes were controlled by two micromanipulators (SM–5 9 Luigi and Neumann). The aCSF/haCSF was continuously pumped via masterflex BPT tubing (Cole-Parmer instrument) through a dual-channel inline solution heater (Supertech) passing a drop chamber before entering the dual-flow recording chamber (Supertech). In the recording chamber the solution flowed on either side of a metal grid supporting the brain slice and then exiting via a drop chamber *(*figure 4; *paper I* and *III-IV*). For experiments without epileptiform activity (*paper II*) a normal recording chamber heated from the holder was used. For the experiments involving optogenetics, *paper I* and *II*, the light was generated by a 460 nm light emitting diod (Prizmatix) and guided through the microscope objective to illuminate the entire slice. The specific light programs with different durations and frequencies were programed and executed with a Master-8 stimulator (AMPI). The mouse slices were recorded for approximately one hour and the human tissue slices were used in the setup for 2-5 hours. For fixation, the recorded slices were placed in 4 % paraformaldehyde (PFA), refrigerated overnight and rinsed the next day for further immunohistochemical analysis.

## Recording in mouse hippocampal slices

In the work presented in *paper I* whole-cell recordings were mainly performed on principle cells in subiculum but also on PV-interneurons. Whole-cell recordings in slices obtained from CK-ChR2 mice were made with a pipette solution containing (in mM): 122.5 K-gluconate, 12.5 KCl, 10 KOH-HEPES, 0.2 KOH-EGTA, 2 Mg-ATP, 0.3 Na3GTP, and 8 NaCl, (pH 7.2–7.4, osmolarity 290–300 mOsm), while for whole-cell recordings in slices obtained from PV-ChR2 mice, the pipette solution contained (in mM): 140 K-gluconate,

10 KOH-HEPES, 0.2 KOH-EGTA, 2 MgATP, 0.3 Na3GTP, 4 NaCl (pH 7.4 , osmolarity 292 mOsm). When a successful whole-cell configuration had been established using the patch-clamp technique, the resting membrane potential was recorded in current-clamp mode. Followed by a series of depolarising steps to reach the cells action potential threshold. The experimental protocol with light stimulations was then performed and the cells response recorded in current-clamp mode. In a subset of experiments a simultaneous field recording was conducted in CA1 (stratum radiatum). The purpose of this recording was to see if certain activity recorded from the cells in subiculum also could be detected in the field, confirming a network response. The convulsive agent 4AP was added to the aCSF to enhance background activity in a group of experiments.

## Recording in human hippocampal slices

The human hippocampal and cortical organotypic culture slices used in *paper II* were cut from the cell culture insets and placed submerged, with the cell culture inset-membrane side down, in a normal recording chamber, accessing the cells from the top of the slice. The cells expressing ChR2 was identified by activation of the GFP tag, expressed with the opsin, by illumination of blue light. Whole-cell patch-clamp recordings was attempted on the identified cells and when successful the intrinsic properties such as resting membrane potential and ability to generate action potentials was recorded in current-clamp mode. During experiment the cells response to blue light was recorded in both current-clamp and voltage-clamp, and glutamate-receptor blockers (AP5, NBQX) and $GABA_A$-receptor blocker (PTX) was added to clarify how much of the cellular response was due to light activation of ChR2.

For the work presented in *paper III* and *IV* the human hippocampal slices were moved to the dual-flow recording chamber after 3 (*paper III*), 24 (*paper III* and *IV*) or 48 (*paper III*) hours of interface incubation. Whole-cell recordings were performed on granular cells in the dentate gyrus easily identified from their rounded shape and placement close to each other in the cell layer. In a subset of experiments, a field electrode was placed within the granule cell layer to record network activity. Intrinsic properties such as resting membrane potential, action potential generation during depolarising steps and a baseline recording in current-clamp mode revealing spontaneous postsynaptic potentials was performed. In the experiments for *paper III,* we used 0Mg haCSF alone or in combination with 4AP. In *paper IV* we used 0Mg haCSF in

combination with 4AP to induce a baseline of epileptiform activity on which the effect of NPY on its own or together with the Y2 receptor antagonist BIIE0246 was tested.

For all whole-cell patch-clamp recordings in human tissue a pipette solution containing (in mM): 122.5 K-gluconate, 12.5 KCl, 10 KOH-HEPES, 0.2 KOH-EGTA, 2 Mg-ATP, 0.3 Na3GTP, and 8 NaCl (pH 7.2–7.4 osmolarity 290–300 mOsm), was used.

# Immunohistochemistry

When performing whole-cell recordings biocytin was added to the pipette solution to infuse into the recorded cell for later identification. Recorded slices, fixed overnight in 4 % PFA were either rinsed and stained immediately or placed in Walter's antifreeze solution and stored at -20˚C to be stained at a later time point. Depending on the purpose of the staining the slices was either subsliced on a sliding microtome (Microm HM 440 E) to 30 µm thick slices or stained without subslicing (400 µm thick). The slices were rinsed 3 x 10 min in potassium phosphate buffer solution (KPBS) prior to blocking with either 5-10% normal goat serum or normal donkey serum in 0.025% Triton X-100 KPBS and incubated with primary antibodies overnight at room temperature (30 µm thick slices) or at 4˚C for 48 hours (400 µm thick slices). The slices were then rinsed again, incubated with the appropriate secondary antibody for 2 hours followed by one last rinsing step before mounted on glass slides using Dabco, with or without Hoechst.

For the viability assessment in *paper III,* human hippocampal slices were fixed in 4% PFA after 0, 3, 24, and 48 hours of incubation (no electrophysiological recordings were performed in these slices). The slices were further sectioned to 20 µm thickness, using a cryostat (Cellab Nordia AB), and directly mounted on glass slides (+charged Menzel-Glas, Thermo Scientific). The slides were stored at -20˚C until staining. The same staining procedure as previously described for thin slices was then followed, with the exception that the slices were mounted instead of free-floating.

Post-staining evaluations and images were acquired using an epifluorecence microscope (Olympus BX61) or a confocal microscope (Inverted Nikon Eclipse Yi microscope Csi). Please see each paper for information on specific antibodies and their concentrations.

# Data analysis and statistics

Analysis of electrophysiological data was performed with Fitmaster (HEKA), Igor Pro (Wavemetrics) and MiniAnalysis (Synaptosoft). For analysis of images Fiji/ImageJ (https://imagej.nih.gov/) was used.

To statistically analyse the data from a normally distributed population the paired *(paper III)* and ratio paired *(paper IV)* Student t-test was used when comparing two groups and one-way or repeated measures ANOVA *(paper III)* followed Turkey's post-hoc test for multiple comparison or Bonferroni correction when comparing three groups. For data non-normally distributed, Kruskal-Wallis test for, no matched or paired data, and Friedman's test for, matched or paired data, was used for comparing three groups *(paper III).* The Fishers exact test was used for comparing two groups responding to two treatments *(paper I)* and the Kolmogorov Smirnov test was used for analysis of cumulative distributions *(paper III and IV)*. All statistical analysis were performed with Prism (GraphPad 7) with the significance level set to $p < 0.05$.

# Results

The overall aim of this thesis was to validate experimental data from animal models in human epileptic brain tissue, with the specific focus on the role of different neuronal populations in seizure initiation, as well as develop and use this platform in validating novel anti-seizure treatment strategies.

## The role of different neuronal populations in seizure initiation

Epileptiform activity *in vitro* is usually generated in hippocampal slices from normal animals by changing the ionic composition of the aCSF, or by adding a drug, such as 4AP. We know, however, that epilepsy and seizures will have consequences for the brain, e.g. changing neuronal connections (see introduction) making the chronic epileptic brain fundamentally different from a naïve, albeit hyperexcitable, one. To attempt to address this discrepancy, our initial goal was to investigate the role of different populations of neurons in seizure initiation in an established epileptic network (*paper I*). This was done by synchronising inhibitory or excitatory populations of neurons to generate epileptiform activity, using optogenetic techniques, in hippocampal slices obtained from epileptic and normal mice.

### Epileptiform activity was only initiated in the presence of 4AP

Previous studies have demonstrated the possibility to generate and sustain epileptiform activity by activation of specific neuronal populations such as PV-interneurons (Sessolo et al. 2015, Shiri et al. 2015, Yekhlef et al. 2015). We investigated if epileptiform activity could be triggered without using 4AP, but instead by activation of specific neuronal populations in an endogenous epileptic hippocampal slice. For this study we used the CK-ChR2 mouse strain

with ChR2 expressed in excitatory cells under the CamKIIa promoter and the PV-ChR2 mouse strain with ChR2 expressed in PV-interneurons. SE was induced by systemic KA injections, resulting in changes associated with TLE. Hippocampal slices from epileptic animals and normal animals were obtained to investigate if epileptiform activity could be triggered by optogenetic activation of either excitatory neurons or PV-interneurons.

In whole-cell recordings from subiculum, the hippocampal output structure, neither activation of excitatory cells nor activation of PV-interneurons could generate epileptiform activity in slices from epileptic animals. Spontaneous PDSs were detected both when activating excitatory neurons and PV-interneurons. This was not exclusive for epileptic mice but was also seen in slices from normal mice.

If 4AP was added to the aCSF it was possible to generate epileptiform ADs by activation of excitatory neurons. The ADs were similar to what have previously been reported by our group *in vivo* (Berglind et al. 2018) and by others *in vitro* (Ellender et al. 2014). ADs were detected both in the whole-cell patch-clamp recordings in subiculum and in simultaneous field recordings from the CA1, implicating the ADs were a network phenomenon and not just present in individual cells in subiculum. To get some clues about the mechanism behind the ADs triggered by activation of excitatory cells in the presence of 4AP, we continued by blocking $GABA_A$ receptors. This resulted in a complete abolish of ADs previously seen after the light stimulation train.

To investigate the role of different neuronal populations in seizure initiation in the most relevant model, epileptic tissue from patients, using optogenetics, we needed to extend the lifespan of human slices to allow viral vector expression in specific populations of neurons. To achieve this goal, we considered two alternatives: (i) to culture human hippocampal slices for several weeks, and (ii) to optimise acute slice incubation conditions so that acute slices would remain viable for at least 2 days. These studies were performed and described in *paper II* and *III*.

# Proof-of-concept, optogenetic tools can be used in human brain tissue

Optogenetics has proven to be a useful toolbox for neuroscientists in exploring and understanding the brain and its diseases. But it is also an interesting technique from a treatment perspective. If we can control neurons with light maybe we can take control over neurological diseases and disorders. To investigate if optogenetics can be used to understand more of the seizure generating process in human tissue as well as the use in future antiepileptic treatments we explored the possibility to express ChR2 in human neurons in *paper II*.

### Functional ChR2 expressed in human neurons

A lenti viral vector containing the ChR2 gene under the human synapsin promoter was applied to human hippocampal and cortical slices 12 hours after slicing, followed by placing slices in a standard incubator for organotypic culturing. Two weeks after viral application the organotypic cultures were transferred to the recording chamber in the electrophysiological set up for evaluation of ChR2 function and to see if the expression had jeopardised cell integrity. The whole-cell patch-clamp recordings showed that ChR2 induce fast and strong depolarisation, resulting in action potentials, when activated by blue light. The recordings also show that the neurons expressing the ChR2 have preserved intrinsic properties, indicating that ChR2 can be successfully expressed in human neurons without obvious threat to neuronal function.

# Development of a human tissue test-platform

Most studies investigating epileptiform activity in human slices have used the acute slice preparation and an interface recording setup. Traditionally, we and others, have only kept the human tissue in an acute state up to 20 hours, limiting the time available for performing experiments or expressing viral vectors. With the goal of dissecting seizure-initiation using optogenetic tools, an acute human tissue platform allowing slices to be kept viable for at least 48 hours would be advantageous. This, as well as the possibility to generate

epileptiform activity in a submerged human hippocampal slice, to enable visually guided whole-cell patch-clamp technique, was addressed in *paper III*.

## Prolonging lifespan of human acute hippocampal slices

The incubation setup was comprised of cell culture inserts positioned on the surface of a continuous flow of oxygenated aCSF in an open Plexiglas box. The setup is placed in a closed Plexiglas box with aCSF continuously oxygenated inside the closed box to create a humid atmosphere. The slices were placed on the membrane with access to nutrients in the aCSF flow from underneath and a maximised access to oxygen from the humid air around, see figure 4. The slices were incubated in the interface setup at room temperature for 3, 24 or 48 hours. No changes were detected between the three time points in regard to cell morphology or apoptosis, shown by immunohistochemical staining for MAP2 (for visualisation of the cells morphological structures), NeuN (for counting positively labelled cells in the dentate gyrus) and Cas3 (for counting of apoptotic cells). To further investigate the viability of the incubated slices we performed whole-cell patch-clamp recordings in the dentate gyrus. The electrophysiological data confirm that granule cells from slices of all time points were viable with no difference in number or amplitude of spontaneous synaptic events, nor in action potential threshold, amplitude or frequency. Cells from all three time points also displayed healthy resting membrane potentials even if a small difference was detectable between the 3 and 48 hour group.

## Epileptiform activity in a submerged recording chamber

The aim for this part of the project was not only to prolong the incubation of acute human hippocampal slices but also to readily generate epileptiform activity. It has been a general consensus in the research field, working with electrophysiological techniques on resected human tissue slices, that keeping the slices in an interface manner during resting and recording is vital if interest lies in studying epileptiform activity (Jones et al. 2016). By using a dual-flow recording chamber, with a continuous flow of aCSF above and beneath the slice, enabling visually guided whole-cell patch-clamp technique, we were able to generate epileptiform activity in the dentate gyrus with 0Mg-4AP aCSF. Spontaneous epileptiform activity was also recorded in normal aCSF from slices incubated for 3 and 48 hours.

# NPY supress epileptiform activity in human brain slices

Several studies with both *in vitro* and *in vivo* animal models of epilepsy have shown that NPY reduces epileptiform activity and slows down epileptogenesis. In *paper IV* we wanted to use the test-platform optimised in *paper III* to investigate if NPY has a seizure suppressant effect in human drug-resistant tissue.

## NPY reduce the number of PDSs and action potentials

We took advantage of the human tissue test-platform developed in *paper III,* utilising 24 hours of incubation and 0Mg-4AP aCSF to generate robust epileptiform activity, to test if NPY would have a seizure suppressant effect. With whole-cell patch-clamp recordings we could see a reduction in epileptiform activity. When NPY was added to the perfusion medium we detected a decrease in the number of PDSs and action potentials. Also, the time interval between action potentials was longer when NPY is added. In a subset of cells the recordings were stable enough to perform a washout. When the NPY was washed out the seizure reducing effect seen previously was eliminated and no difference in number of PDSs or action potentials were detected between the baseline recording (before NPY is added) and the washout recording (after NPY is washed out).

## Y2 receptor antagonist counteract the effect of NPY

After seeing the seizure suppressant effect of NPY in the human tissue hippocampal slices, we were curios to continue investigating the mechanism behind this. Previous studies using animal models have shown that the Y2 receptor is likely to mediate the seizure suppressant effect of NPY (El Bahh et al. 2005, Nikitidou Ledri et al. 2016, Vezzani et al. 2002, Woldbye et al. 2010). Our group has also reported that application of a specific Y2 receptor antagonist counteracted the observed reduction in excitatory postsynaptic potentials induced by NPY application in human hippocampal slices (Ledri et al. 2015). To test if the seizure suppressant effect of NPY was mediated mainly by the Y2 receptor we added the specific Y2 receptor antagonist BIIE0246 together with NPY. No reduction in epileptiform activity was detected in

experiments with both NPY and the Y2 receptor antagonist, verifying that the Y2 receptor is the main receptor responsible for the anti-seizure effect of NPY.

# General discussion

In this thesis, I first demonstrated that in epileptic hippocampal slices from animals, synchronisation of PV-interneuron or principle neuron activity, by optogenetic stimulation, failed to induce epileptiform afterdischarges. Increasing network excitability by adding 4AP to the perfusion medium did not promote the induction of such activity. On the other hand, optogenetic synchronisation of principle neurons in 4AP conditions did generate epileptiform afterdischarges, most likely by recruiting downstream population of inhibitory interneurons not restricted to just PV-interneurons. To validate whether similar situation holds true in human epileptic tissue slices, first we demonstrated that optogenetic tools could be applied to human epileptic brain slice cultures. The culturing was required to prolong the lifespan of the slices to allow viral-vector based opsin expression. As an alternative to culturing, we next demonstrated that acute human brain slices can be maintained viable for prolonged periods by optimising incubation procedures, to allow viral vector expression. Finally, we validated that the anti-seizure effect of NPY, previously demonstrated in several animal models, also is seen in epileptic human brain tissue. Slice culturing may not be an optimal approach since it could induce further neuronal network reorganization, which is not desirable in already epileptic tissue. Therefore a follow-up of these studies should be, to selectively express optogenetic tools in the acute slice platform, to test whether findings in animal *in vitro* model concerning the role of different neuronal populations in generating epileptiform afterdischarges also holds true for epileptic human tissue.

## Which neuronal population participate in seizure initiation

Selective optogenetic activation of excitatory neurons or PV-interneurons did not result in epileptiform activity in subicular slices from epileptic or normal

mice. With an increased excitability, in non-epileptic mouse slices, induced by 4AP, optogenetic activation of excitatory cells but not PV-interneurons resulted in ADs. This finding suggests that activation of exclusively excitatory cells or PV-interneurons will not be enough to generate epileptiform activity even in a hippocampal slice from an epileptic animal. It is however possible to generate epileptiform activity (in the form of ADs) by selective optogenetic activation of excitatory cells *in vivo* without a chemically induced increased excitability (Berglind et al. 2018). This *in vivo* study also demonstrates that the engagement of the contralateral (to the optogenetic light stimulation) hippocampus is important for the development of epileptic ADs. In an *in vitro* setup the remote neuronal network of a slice is very limited compared to the *in vivo* situation and this could be a plausible reason for the inability to optogenetically trigger ADs *in vitro*.

Other *in vitro* studies have observed that optogenetic activation of PV-interneurons in the presence of 4AP can trigger (Shiri et al. 2015, Yekhlef et al. 2015) or propagate (Sessolo et al. 2015) epileptiform activity, demonstrating the important role of interneurons during seizure generation. In contrast to previous studies, we could not trigger epileptiform activity in subiculum by activating PV-interneurons in the presence of 4AP. We could show, however, a clear GABAergic dependence in the ADs triggered by optogenetic stimulation of excitatory cells.

Previous *in vitro* studies focused on the medial entorhinal cortex, while our recordings are made in the subiculum. It would be interesting to further compare the micro network differences in these two areas to see if that could explain the different type of activity observed when activating PV-interneurons. In a recent study Shiri et al. 2017 showed that low frequency optogenetic stimulation of principle cells suppressed 4AP induced epileptiform activity longer than activation of PV-interneurons did. They suggest that interneurons in the medial entorhinal cortex target a large local population of principle cells causing a large rebound effect, while post-excitatory inhibition triggered by targeting principle cells is expected to be larger due to larger field responses evoked by principle cells compared to interneurons (Shiri et al. 2017). Further, the strong entorhinal cortex – hippocampus connection play an important role in triggering or silencing epileptiform activity. Principle cells in the entorhinal cortex have direct connections to interneurons in the hippocampus and optogenetic stimulation of principle cells in entorhinal cortex leads to a reduction of epileptiform activity in hippocampus (Canto et al. 2008, Shiri et al. 2017, Xu et al. 2016). The inability to evoke ictal epileptiform

activity with 4AP and optical stimulation of PV-interneurons in the subiculum could also be due to the variability of GABAergic inhibition within different cell types of the subiculum (Lee & Maguire 2014). The regular spiking cells and interneurons have similar magnitudes of tonic inhibition (Panuccio et al. 2012) while the intrinsic bursting cells are much stronger inhibited (Menendez de la Prida 2003), possibly making it harder to trigger epileptiform activity with global illumination. Future experiments with local light stimulation and electrophysiological recordings performed in both the subiculum and the entorhinal cortex is needed to investigate this further.

## Optogenetic tools in human brain tissue

We show in *paper II* that optogenetic tools such as ChR2 can be expressed in human tissue and can be used to manipulate neurons without compromising the intrinsic properties of the cell. The results provide an important proof-of-concept, showing that it is feasible to use optogenetics in human brain tissue. This opens for the technique to be used in exploration of neural network functions in human brain tissue *ex vivo* and to continue the work in *paper I* by exploring the roles of different neuronal population in seizure initiation. It also opens for a possible future optogenetic therapeutic approach in neurological diseases.

Our results were obtained by using a lenti viral vector to infect tissue resected as a result of brain surgery, but using another type of virus is likely feasible as well. In post mortem brain tissue, obtained by autopsy within 8 h after death, the adeno associate virus was successful in infecting cells in tissue cultured up to four weeks (Verwer et al. 2002). Optogenetics has successfully been used in research with non-human primates, the first studies mainly focused on mechanisms within the visual system (Han et al. 2009) but the work has since then expanded, targeting several brain areas to gain new insight in the function of specific neural circuits (Galvan et al. 2017, Tamura et al. 2017). The non-human primate studies have also revealed several technical problems with using optogenetics in larger animals with a brain more like our own. These problems are mostly due to the larger size of the brain, making viral vector and light delivery more challenging. But also promoter specificity, ensuring the opsin is only expressed in the targeted neuronal group have proven to be a challenge (Galvan et al. 2017). To date no gene therapy treatments have been tested in patients with epilepsy, but promising results from clinical trials testing

gene therapy treatment using viral vectors in for example patients with Parkinson disease show a therapeutic effect as well as being safe (Marks et al. 2016, Piguet et al. 2017, Simonato et al. 2014). Using resected human brain tissue to continue exploring viral vector expression of optogenetics could be a good compliment to the non-human primate studies in overcoming future technical challenges of using optogenetics in humans.

# The human tissue test platform – bridging the gap between animal models and the clinic

By using an interface incubation setup we show that resected human hippocampal slices can be kept as acute slices for at least 48 hours without obvious signs of reduced viability. In the electrophysiological experiments we used a dual-flow recording chamber, enabling visually guided whole-cell patch-clamp technique, showing evoked epileptiform activity is possible in the submerged slices. The method presented in *paper III* opens up for prolonged testing of new anti-seizure treatments including expression of certain viral vector types in human brain tissue. This test-platform can be used as an intermediate step between animal models and the clinic to study new treatment options (see figure 6) but also to gain more knowledge about the mechanisms behind seizure generation. Using the dual-flow recording chamber, previously only used in experiments with rodent slices, facilitate data collection from individual cells during epileptiform activity by enabling visual guidance for the patch-clamp technique.

In previous electrophysiological studies using human hippocampal slices the acute incubation time reported is between 1 and 20 hours (Hsiao et al. 2012, Jones et al. 2016). The majority of previously published electrophysiological data displaying epileptiform activity comes from field recordings. With three studies showing epileptiform activity in dentate gyrus evoked by electric stimulation in high potassium aCSF (Gabriel et al. 2004, Jandová et al. 2006) and high potassium – low magnesium aCSF (Eugene et al. 2014). To evoke epileptiform activity during electrophysiological recording we excluded magnesium and added 4AP in the aCSF. We have so far not thoroughly investigated other methods to evoke epileptiform activity but see no reason why methods tested by other groups (Jones et al. 2016) in the interface recording chamber would not work in our set up as well.

The study focused on the dentate granule cells, partially due to practical reasons, with the slices not always including the whole hippocampal formation – but always dentate gyrus, but also because of the gating function of dentate gyrus within the hippocampus. Thus we cannot say if the epileptiform activity was exclusively evoked there or if other regions within the slice also displayed epileptiform activity. This question is yet to be answered in the acute slice setup but a spread from the dentate gyrus to the rest of hippocampus is likely as it has been shown to occur in organotypic human hippocampal slices (Eugene et al. 2014).

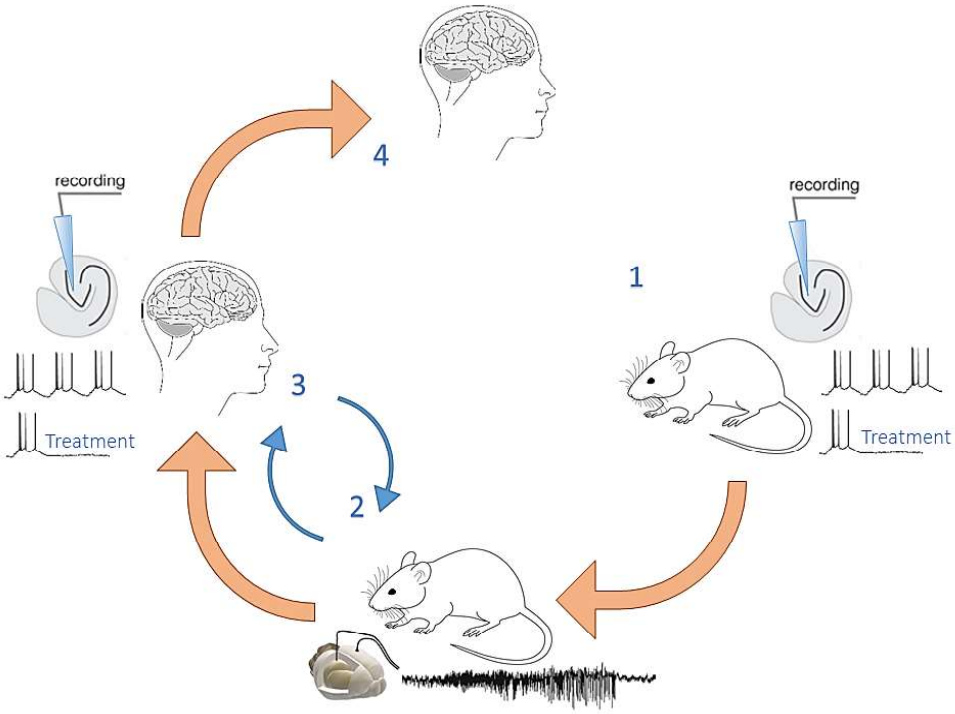


**Figure 6: Human tissue – bridging the gap between animal models and the clinic.**
Findings of new drugs and treatments are usually made in animal models (1, 2). Using human tisue slices from drug-resistant epilepsy patients to test the functionality of the drug or treatment is one way to bridge the gap between animal models and the clinic (3, 4).

Prolonging the timespan for slices to be kept in an acute incubation setup even longer than 48 hours will be companied by a high risk of bacteria growth. We reduced the accumulation of bacteria by regularly changing the aCSF and including a UV-C illumination step before the aCSF is pumped into the slices. In a setup for rodent slices UV-C illumination and cooling the aCSF have been shown to prolong the incubation time with preserved viability up to 36 hour in rat slices (Buskila et al. 2014). To keep slices viable longer than 48 hours the

organotypic culture method is a good option. So far only one publication has provided data showing epileptiform activity can be recorded from human hippocampal organotypic slices (Eugene et al. 2014). But the method could possibly be improved by using human CSF, recently demonstrated in resected human neocortical slices (Schwarz et al. 2017).

We have in, contrast to what has previously been published (Cohen et al. 2002, Huberfeld et al. 2007), detected spontaneous epileptiform activity (in normal aCSF) in the dentate gyrus. Spontaneous SLE and interictal-like epileptiform activity in the hippocampus have previously only been reported in CA2 and subiculum (Cohen et al. 2002, Huberfeld et al. 2007, Wittner et al. 2009). Taken together it is now demonstrated that the three areas of the hippocampus (subiculum, CA2 and dentate gyrus), that during epilepsy are less prone to cell death, can generate epileptiform activity spontaneously. Our recordings of spontaneous epileptiform activity also give an indication that the slices do get enough oxygen in the dual-flow recording chamber and that it is comparable with the interface recording chamber.

## Validating the results from animal models in human tissue

By using the human tissue test-platform developed in *paper III* we show that application of NPY supress epileptiform activity in the dentate gyrus of human hippocampal slices resected from patients with drug resistant epilepsy. Our hypothesis, that the anti-seizure effect is mediated mainly via the Y2 receptor was confirmed by lack of effect in the presence of an Y2 receptor antagonist. The results in *paper IV* validate that NPY, a peptide tested in several animal models of epilepsy and put forward as a promising future drug candidate, can supress epileptiform activity in human brain tissue. This finding suggests that increased levels of NPY could have a seizure-suppressant effect in patients that are not responding to today's anti-epileptic drugs.

The seizure-suppressant effect of NPY has previously been documented in several *in vitro* studies using rodent slice seizure models (Bijak 1999, El Bahh et al. 2005, Klapstein & Colmers 1997, Woldbye et al. 2002). Two studies, both using 0Mg aCSF to generate epileptiform activity in mouse hippocampal slices reported a 40 % reduction in bursting frequency, a result that is

comparable with the reduction of PDSs reported in *paper IV* (Bijak 1999, El Bahh et al. 2005).

The results from *in vitro* rodent studies have been confirmed in rodent studies *in vivo.* These studies have demonstrated that overexpression of NPY in acute and chronic seizure models have a seizure-suppressant effect (Deborah Lin et al. 2006, Foti et al. 2007, Noe et al. 2010, Noè et al. 2008, Richichi et al. 2004, Sørensen et al. 2009). Several studies also show that in hippocampus the Y2 receptor plays a major role in mediating this effect of NPY (El Bahh et al. 2005, Nikitidou Ledri et al. 2016, Vezzani et al. 2002, Woldbye et al. 2010).This theory, developed through animal model studies is now supported by the results from human hippocampal tissue in *paper IV*.

Upregulation of Y2 receptor have also been observed in both rodents and humans in relation to epilepsy and seizures (Furtinger et al. 2001, Gobbi et al. 1998, Roder et al. 1996), which could be seen as an endogenous anti-seizure mechanism (Vezzani & Sperk 2004).

It is important to remember that in our *in vitro* experiment we have not measured any long-term effects of NPY application. NPY is an endogenous peptide with several receptors, which has an active role in many functions in the brain, making it possible that homeostatic changes may occur in response to elevated levels of NPY. But the effects of increasing NPY in an epileptic brain can be different from the effects seen in a healthy brain. This was highlighted by Sørensen et al. in 2009 Showing that a negative effect on the hippocampal memory function due to increased levels of NPY was only observed in healthy animals and no such changes was detected in the epileptic animals (Sørensen et al. 2008, 2009).

The results from *paper IV* validate, in human hippocampal slices, what has previously been observed in several animal models of epilepsy *in vitro* and *in vivo*. Taken together this further supports the development of novel treatment strategies using gene therapy to increase expression of NPY and its Y2 receptor for patients with drug-resistant epilepsy. The results also confirm that the human tissue test-platform can be used for testing new drugs and treatments. The next step for further development of this platform would be to express optogenetic tools to continue investigating different neuronal population roles in seizure initiation in a human epileptic network as was done in animal model in *paper I*.

# Future perspective

To continue the work with generating the type of preclinical data that will motivate and empower more successful clinical trials we need to work with models that are clinically relevant. Good *in vitro* models are important for drug screening and to understand the mechanisms behind a new treatment or drug. Testing new a treatment or a drug in human tissue would give additional information that could prove essential for a go or no-go decision at the start of a clinical trial, potentially saving money and effort.

There are still many un-walked paths to explore when working with human tissue, in both hippocampus and cortex. Only a few studies have so far used a multi electrode array (Dossi et al. 2014, Hsiao et al. 2015) to look at epileptiform activity over a larger area. To continue and study human tissue with this approach would be exciting, especially to learn more about how epileptiform activity propagates, and learn clues that might help us stopping it. It would also be interesting to continue investigating the different roles played by excitatory and inhibitory neurons during seizure initiation and propagation, by using the high spatial and temporal precision provided by optogenetic tools. The same tools can also be used in human tissue to explore the possibility of stopping epileptiform activity, and eventually seizures *in vivo*, by controlling certain populations of neurons with light. With the results obtained in *paper IV*, showing that epileptiform activity is reduced by NPY activation of the Y2 receptor, aids in validating a future gene therapy approach using a NPY-Y2-vector. It has recently been demonstrated that human cells release more NPY after successful transduction with an viral vector-NPY construct (Patrício et al. 2018). The next step would be to see if it is possible to reduce epileptiform activity by introducing a transgene expression of NPY and Y2 receptor in human slices with a gene therapy approach. One could further envision a scenario where local gene therapy is targeted to a brain tissue area, planned to be resected in a patient with drug-resistant epilepsy, prior to resection. If the treatment is not working or displays unexpected side effects, the resection can continue as planned and the treated tissue will be removed.

The future perspective of preclinical research is to develop a preclinical road-map, choose the best therapeutic candidates, and reduce the risk for pharmaceutical companies in taking on development of new AEDs, leading to more therapeutic alternatives reaching the clinic. For this to happen, a joint effort with more multicentre preclinical trials carefully designed to generate data translatable to the clinic is the way forward.

# Concluding remarks

Neuroscience research is moving forward with new discoveries and tools that help us understand the function of the brain and how to solve or aid dysfunction. For obvious reasons, most research is not performed in humans but animals, cell culture models and computer models are used instead. With every discovery made, we have to remember it only holds true for that specific model until we can show otherwise. To move from a discovery in a rodent to testing in humans has proven difficult. Many drugs show promising results in animal models but fail in the early clinical trials. This is of course a problem that needs to be addressed from several angles and there is no simple solution. In this thesis, I show that one angle of help to overcome this problem is the use of resected human brain tissue from patients with epilepsy. The work accomplished in this thesis has moved the field forward by prolonging the use of this unique tissue and by verifying that NPY, a peptide with good results from animal models, has a similar effect in resected human brain tissue. A finding that takes this potentially new treatment one step closer to a successful clinical trial.

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# Appendix

Paper 1

Paper 2

Paper 3

Paper 4

# Paper I

# Paper II

SCIENTIFIC REPORTS

OPEN

# Optogenetic control of human neurons in organotypic brain cultures

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**Optogenetics is one of the most powerful tools in neuroscience, allowing for selective control of specific neuronal populations in the brain of experimental animals, including mammals. We report, for the first time, the application of optogenetic tools to human brain tissue providing a proof-of-concept for the use of optogenetics in neuromodulation of human cortical and hippocampal neurons as a possible tool to explore network mechanisms and develop future therapeutic strategies.**

Optogenetic tools are used in an increasing number of studies and have successfully resolved many unknown details related to the role of different populations of neurons in mechanisms governing normal brain function, such as learning and memory[1,2], circadian rhythms[3–6] and visuospatial discrimination[7]. Moreover, successful attempts have been made at understanding the pathophysiology of neural diseases in animal models, and possibilities for prosthetic correction of impaired brain function by diverse optogenetic approaches[8–10]. However, until now, it still remained unclear whether an optogenetic approach would be feasible in human brain tissue as an important step towards developing alternative treatment strategies for severe neurological diseases. Here we demonstrate that neurons in brain tissue derived from adult human temporal lobe neocortex or hippocampus resected because of medically intractable epilepsy, are capable of expressing ChR2, one of the main excitatory opsins widely used for optogenetic studies in animals. Furthermore, we show that human neurons expressing ChR2 are activated by blue light to generate action potentials.

Temporal neocortical (NC) tissue and human hippocampus (HPC) were obtained by surgical resections (4 NC/4 HPC) from seven patients treated for intractable epilepsy at the Department of Neurosurgery of Lund University Hospital, Sweden, and Rigshospitalet in Copenhagen, Denmark. The procedures and use of resected human brain tissue were approved by the local Ethical Committee in Lund, (#212/2007) and Copenhagen (H-2-2011-104) in accordance with the Declaration of Helsinki and written informed consent were obtained from all subjects prior to surgery. Neocortex or hippocampus were surgically removed *en bloc* and immediately submerged in carbonated ice-cold sucrose cutting solution and transferred from the operating room to the laboratory, where 250 μm thick slices of temporal lobe neocortex and the hippocampal formation were prepared and cultured under standard slice culturing conditions[11]. The slices were allowed to settle for 12 h, and thereafter lenti-viral vector containing ChR2 gene under the human synapsin promoter (LV-Syn-hChR2(H134R)-eYFP) was added. See supplementary information for extended materials and methods.

After two weeks of culturing, slices were transferred to the recording chamber and ChR2 transduction of neurons in the human organotypic brain cultures was identified by expression of eYFP, which was attached as a label to the ChR2 viral vector construct (Figs 1a–c and 2a,b). The ChR2 expression was widespread, covering most of the cultured slice areas, and was localized in cells with neuronal appearance, i.e. clearly identifiable soma and dendrites and co-stained for microtubule-associated protein, MAP2 (Fig. 1a–c). Quantification of the percentage of cells co-expressing MAP2 and eYFP in 23 cultured slices revealed that a large portion of these cells were neuronal ($60.4 \pm 3.9\%$ of eYFP positive cells).

Whole-cell patch-clamp recordings were performed from eYFP-expressing neurons (Fig. 2a,b) to test for functional properties and functional state. The majority of the recorded neurons (45 of 63) showed a resting

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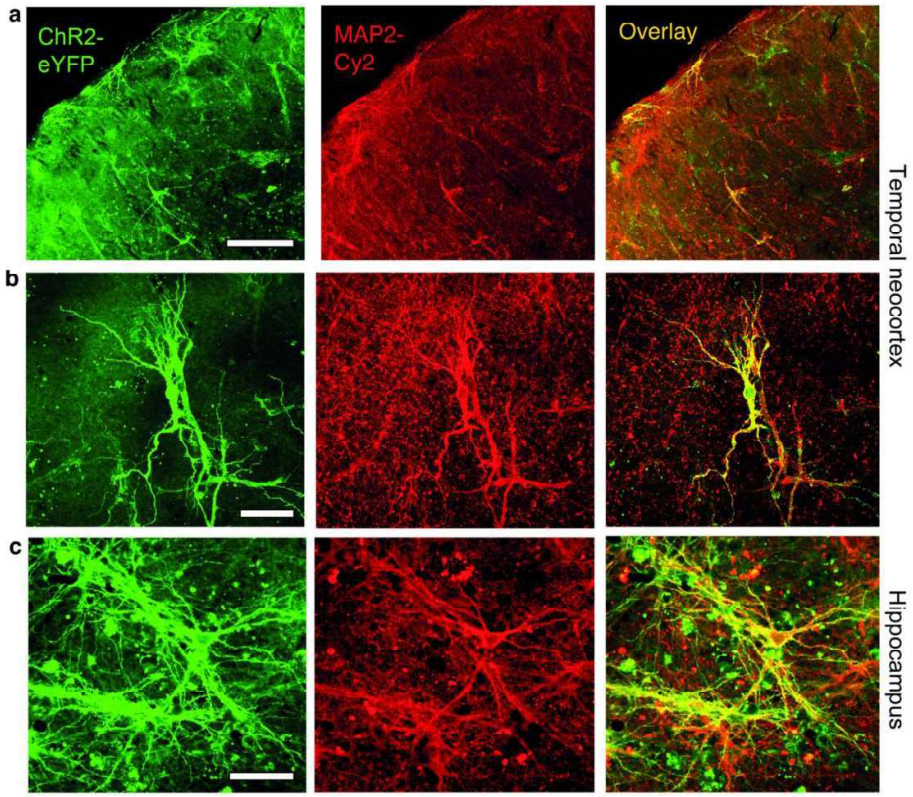


**Figure 1. ChR2 expression in human organotypic brain slice cultures.** Representative confocal images of enhanced yellow fluorescent protein expression (eYFP, green), neuron-specific microtubule associated protein-2 (MAP2, red) and the overlay of both channels. (**a**) Left, middle and right image from a cortical organotypic tissue culture (scalebar 200 µm) with a higher magnification image in (**b**) (scalebar 50 µm). (**c**) Left, middle and right image from a hippocampal organotypic tissue culture (scalebar 50 µm).

membrane potential more negative than −45 mV, measured immediately after breaking into the whole-cell mode. This is in line with our previous recordings from neurons in acute temporal cortical slices[12]. Neocortical and hippocampal neurons did not differ from each other with regard to the magnitude of resting membrane potential (−60.2 ± 2.7 mV, n = 24 and −61.5 ± 2.2 mV, n = 22, respectively, P = 0.71) nor in their input resistance (207 ± 43 MΩ, n = 24 and 232 ± 29 MΩ, n = 23, respectively, P = 0.46). Twenty-one neurons of 45 generated action potentials when step-depolarized by current injection, with average amplitude of 54.0 ± 17.3 mV and duration of 3.1 ± 0.3 ms for neocortical cells (n = 13, Fig. 2c) and 34.0 ± 6.2 mV and duration of 3.6 ± 0.5 ms for hippocampal cells (n = 8, Fig. 2d). These characteristics were closely comparable to those of human neurons recorded in acute neocortical[12] and hippocampal slices[13]. Electrical stimulation of CA1 stratum radiatum fibers induced EPSCs (often poly-synaptic), confirming functional synaptic afferents to these neurons. Taken together, these data show that the majority of the neurons in organotypic cultures were relatively healthy and exhibited normal neurophysiological intrinsic properties.

Since neurons from hippocampal and cortical slice cultures did not differ with regard to their intrinsic properties (P = 0.28 for AP-amplitude and 0.38 for AP-duration), the data on optogenetics from these neurons were pooled together. When slices were exposed to 470 nm-blue LED-light delivered though the 40X-objective, ChR2 expressing neurons responded by depolarization with a typical initial peak current followed by a lower steady-state depolarization (783.9 ± 100.0 pA and 536.5 ± 69.4 pA, respectively, n = 31; see Fig. 2e,g). Moreover, in current-clamp mode, action potentials induced by light stimulation were also frequently observed (Fig. 2f). Repetitive light-pulse stimulations reliably induced depolarizing currents, (827.6 ± 150.6 pA, n = 16, Fig. 3a). Paired-pulse light stimulation with 100 ms interval (with a peak amplitude first response of 952.6 ± 134.7 pA, n = 32) revealed a decrease in amplitude of the second pulse-induced response with the ratio to the first of 0.7 ± 0.1 (n = 32, see Fig. 3b). These currents were mainly generated by ChR2 activation and partly by synaptic inputs from other light-activated neurons, since addition to the perfusion medium of AMPA receptor blocker NBQX and NMDA receptor antagonist AP5 alone, or in combination with $GABA_A$-receptor blocker PTX, resulted in a small reduction of the light-induced currents (to 87.3 ± 4.9%, n = 5, p = 0.04 and 71.5 ± 4.1% of

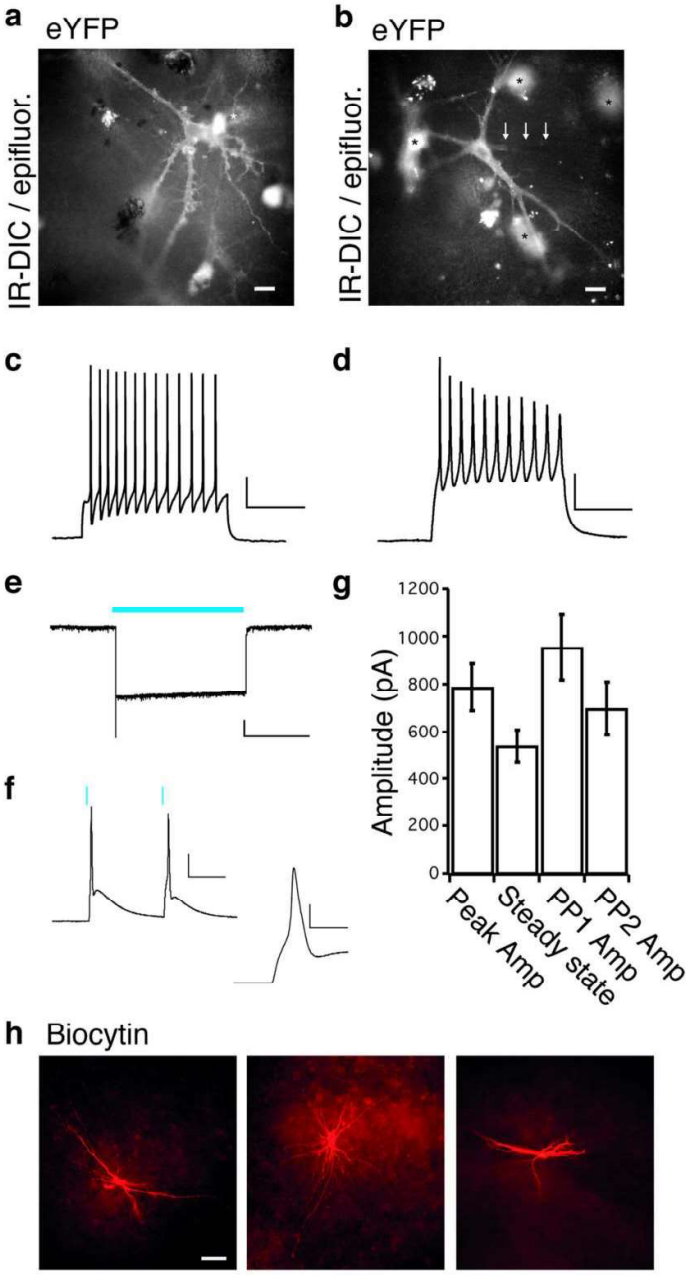


**Figure 2. Light-induced responses of ChR2-expressing neurons in human organotypic brain tissue cultures.** ChR2-expressing cells were identified for whole-cell patch-clamp recordings with the expression of the reporter EYFP in infrared-differential interference contrast (IR-DIC) microscopy combined with epifluorescence exemplified in (**a**) a cultured temporal neocortical neuron and in (**b**) a cultured hippocampal neuron (scale bars 20 μm). Neurons in both preparations, temporal neocortical neuron (**c**) and hippocampal neuron (**d**) fired action potentials in response to a depolarising current step (scale bars 20 mV, 200 ms). (**e**) A 10-s continuous blue light-pulse induced an inward current in voltage clamp (scale bar 50 pA, 5 s) and in (**f**) paired 1 ms light pulses elicited action potentials in current-clamp (scale bar 20 mV, 50 ms and 5 ms in inset). (**g**) Average current obtained from hippocampal and temporal neocortical neurons induced by blue light application, measured as peak amplitude (peak amp) or steady state (2 s after peak) evoked by a 5 s light pulse (n = 31) or peak amplitude in first response (PP1) or second response (PP2) after paired 1 ms light pulses, 100 ms interval (n = 32). (**h**) Biocytin staining showing three representative patterns of dendritic morphology from whole-cell recorded neurons (scale bar 50 μm).

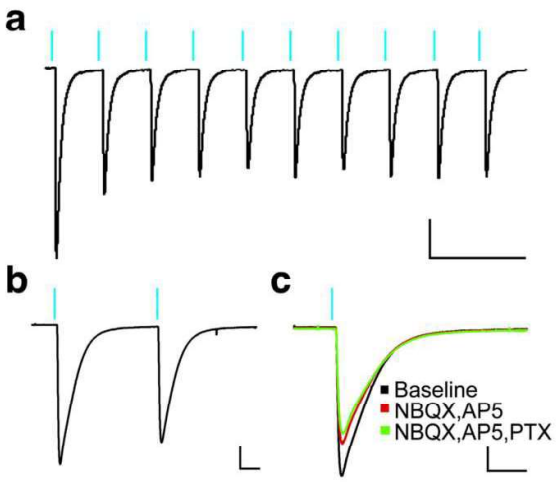


**Figure 3. Light-induced currents in ChR2-expressing neurons in the presence of glutamate and GABA receptor antagonists.** (**a**) A 10-pulse train (1 ms at 50 Hz) of blue light consistently elicited inward currents in voltage-clamp mode. (**b**) Paired-pulse light stimulation with a 100 ms interval (scale bar 200 pA, 20 ms). (**c**) Representative traces showing that a large part of the light-induced currents remained after AMPA (NBQX), NMDA-(AP5) and $GABA_A$-(PTX)-receptor blockade (scale bar 200 pA, 20 ms).

control, $n = 3$, $p = 0.03$, respectively; see Fig. 3c) with no change of the paired-pulse ratio in the presence of antagonists (normalised to baseline, in AMPA and AP5, $99.4 \pm 0.4\%$, $n = 5$, $P = 0.26$ and in AMPA, AP5 and PTX, $99.6 \pm 0.9\%$, $n = 3$, $P = 0.71$), suggesting that reduction of the second light pulse-induced response was mainly due to ChR2 activation/inactivation dynamics[14]. Overall, these data show that (i) neurons in cultured human epileptic tissue slices can survive for prolonged periods, up to 14 days with relatively normal physiological function, (ii) that these neurons can effectively express ChR2 and respond to light stimulation by generation of action potentials, and (iii) these light-generated action potentials can induce synaptic responses in the neighbouring neurons.

Human brain tissue has been shown to survive for several hours after resection as acute temporal lobe or cortical slices[15,16], allowing for electrophysiological investigations of neuronal function. Post-mortem brain tissue in culture has been reported to retain morphological characteristics up to four weeks after culturing and express β-galactosidase delivered by an AAV-vector[17]. Electrical stimulation can induce epileptic activity under slice culture conditions for up to 30 days[18]. However, our study is the first demonstration, to our knowledge, of whole-cell electrophysiological recordings from human epileptic tissue successfully cultured in standard medium for up to 2 weeks. In addition to previous studies, this finding is a significant contribution for establishing long-term survival of human brain slices, which would enable certain experimental approaches, e.g. transgene expression studies. This approach also opens up new possibilities for exploring relatively long-term treatment outcomes, such as for antiepileptic drugs or various other treatment strategies. Most importantly, however, our data demonstrate that viral vector-based optogenetic tools can be used to express opsins, such as ChR2, in human neurons to the level that enables functional manipulation of these neurons. Optogenetics has previously been applied to nonhuman primates[19,20]. In these studies, focus was mainly set on clarifying mechanisms within the visual system[7,21]. Collectively, previous primate studies demonstrate that the optogenetic approach reveals intimate mechanisms of network activity associated with certain behaviour of the neuronal systems, which may be specific to primates or humans. Therefore, our study provides an important proof-of-concept that optogenetics can become a feasible approach to explore human brain tissue *ex vivo*, where networks are at least partially preserved, as well as to develop future alternative therapeutic strategies for neurological diseases, such as e.g. epilepsy.

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## Author Contributions

M.A., M.K. and D.P.D.W. conceived the study. M.A., N.A., A.S., J.W., S.H.C., L.H.P., B.J., S.H.C., J.B. and D.P.D.W. performed research. M.K. and M.A. wrote the manuscript.

## Additional Information

**Supplementary information** accompanies this paper at http://www.nature.com/srep

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# Paper III

SCIENTIFIC REPORTS

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# Prolonged life of human acute hippocampal slices from temporal lobe epilepsy surgery

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**Resected hippocampal tissue from patients with drug-resistant epilepsy presents a unique possibility to test novel treatment strategies directly in target tissue. The post-resection time for testing and analysis however is normally limited. Acute tissue slices allow for electrophysiological recordings typically up to 12 hours. To enable longer time to test novel treatment strategies such as, e.g., gene-therapy, we developed a method for keeping acute human brain slices viable over a longer period. Our protocol keeps neurons viable well up to 48 hours. Using a dual-flow chamber, which allows for microscopic visualisation of individual neurons with a submerged objective for whole-cell patch-clamp recordings, we report stable electrophysiological properties, such as action potential amplitude and threshold during this time. We also demonstrate that epileptiform activity, monitored by individual dentate granule whole-cell recordings, can be consistently induced in these slices, underlying the usefulness of this methodology for testing and/or validating novel treatment strategies for epilepsy.**

Epilepsy is a multifactorial neurological disease characterised by pathological hyper-synchronised activity of neurons manifested as recurrent spontaneous seizures. In the majority of cases, symptomatic treatment by currently available anti-epileptic drugs (AEDs) suppresses seizures. However, in about 30–40% of patients, AEDs are ineffective at controlling seizures, leaving patients with diminished quality of life[1]. In these patients, a treatment option is resection of epileptic tissue, provided the seizure-generating focal area is reliably identified and is located outside eloquent cortex[2]. One of the most common structures for focal seizure origin is the temporal lobe, where resections constitute an effective and relatively low risk treatment for a defined patient population[2]. This therapeutic surgical procedure also provides a unique opportunity for pathophysiological evaluation of human epileptic tissue, including the possibility to maintain tissue as live slices readily accessible for electrophysiological recordings[3,4]. Slice preparations from resected tissue are extremely valuable not only for providing information on pathological network mechanisms of the epileptic brain tissue but also for validating novel treatment strategies developed in preclinical studies using animal models[4–8]. Such a validation step is of particular importance considering that the resected human epileptic tissue is drug-resistant and can give indication if new treatments will be effective against refractory seizures[6,9]. Moreover, at present there are few, if any, drug-resistant animal models of epilepsy, and those that have been developed are extremely time and labour consuming[10].

A number of research groups have studied acute human epileptic tissue slices with epileptic activity induced by various chemical manipulations, such as [0$Mg^{2+}$], high [$K^{-}$] and 4-aminopyridine (4-AP)[7,11,12]. These studies utilised an interface-recording chamber to reliably obtain epileptiform activity[12]. It is generally accepted that the interface-recording chamber provides higher oxygen-levels in the slices, compared to a submerged chamber, and therefore enables neurons to fire action potentials (APs) at higher frequencies for a prolonged period of time, allowing for the generation of seizure-like events (SLE)[12–14]. A major drawback of the interface-recording chamber, however, is that it precludes the visual guidance of a pipette for patch-clamp recording of individual cells in the slices, since water-submerged 20× or 40× objectives cannot be used in the microscope. An attractive alternative to the interface-recording chamber could be the dual-flow system[15] designed to maximise oxygen levels by

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| | Patient | Resistance to >2AEDs | Age at surgery (yrs) | Duration of epilepsy (from onset; yrs) | Seizure frequency (n/mo) | AEDs at surgery | Hippocampal pathology |
|---|---|---|---|---|---|---|---|
| *CPH* | 1 | Yes | 52 | 25 | 1 (KFA) | LTG, LEV | HS |
| *CPH* | 2 | Yes | 54 | 14 | 5 (KFA) | LEV, LTG | HS |
| *CPH* | 3 | Yes | 41 | 34 | 8 (SFA), 5 (KFA) | ZNS, LAC, CLB | HS |
| *Lund* | 4 | Yes | 36 | 35 | 5 | LTG, VPA | HS |
| *CPH* | 5 | Yes | 55 | 19 | 8 (SFA), 4 (KFA) | LEV, LAC | HS |
| *CPH* | 6 | Yes | 31 | 12 | 1 (SFA), 1 (KFA) | LTG, LEV | HS |
| *CPH* | 7 | Yes | 57 | 5 | 9 (KFA) | OXC | HS |
| *CPH* | 8 | Yes | 34 | 8 | 3 (KFA) | CBZ, LEV, LAC | HS |
| *Lund* | 9 | Yes | 28 | 16 | 3 | LTG, LEV | *Normal* |
| *CPH* | 10 | Yes | 18 | 12 | 8 (SFA), 8 (KFA) | VPA | HS |
| *CPH* | 12 | Yes | 44 | 16 | 5 | BV, ZNS, LTG | *Normal* |
| *CPH* | 13 | Yes | 19 | 13 | 6 (SFA) 6 (KFA) | LEV, CBZ | HS |
| *Lund* | 14 | Yes | 23 | 22 | 4 (KFA) | LEV, LAC | HS |
| *CPH* | 15 | Yes | 36 | 26 | 12 (SFA), 12 (KFA) | LTG, LEV | HS |
| Lund | 16 | Yes | 6 | 5 | 30 | LTG, LEV | HS |

**Table 1.** Patient data. Seizure frequency reported by patients in Copenhagen (CPH) reported as simple focal seizures (SFS) and complex focal seizures (CFS). If SFS are not noted the patient does not report experiencing auras/SFS. Abbreviations as follows carbamazepine (CBZ), clobazam (CLB), Brivaracetam (BV), lacosamide (LAC), lamotrigine (LTG), levetiracetam (LEV), oxcarbazepine (OXC), valproate (VPA) and zonesamide (ZNS) and hippocampal sclerosis abbreviated as (HS).

providing a laminar flow of oxygenated artificial cerebral spinal fluid (aCSF) on both sides of the submerged brain slice. This system has been reported to enable recordings of sustained epileptiform activity in acute rodent hippocampal slices, with the advantage of allowing visually guided whole-cell patch-clamp recordings[15,16]. However, this approach has not yet been applied to human brain slices[12]. The objective of the present study was two-fold: (i) to test the hypothesis that incubation time for human brain slices, using the interface incubation can be extended to 48 h without significantly compromising slice quality; and (ii) to establish a dual-flow submerged chamber system, enabling visually guided whole-cell recordings during epileptiform activity in human brain slices. The first objective was motivated by the need to increase yield of data from each occasion when human brain tissue becomes available, as well as to extend the time for allowing viral vector expression and thereby the validation step for gene expression and/or therapy effectiveness in human pharmacoresistant epileptic tissue.

## Material and Methods

Temporal lobe resection tissue blocks were obtained after surgery from 16 patients with pharmacoresistant temporal lobe epilepsy (seizures for 5 to 35 years and ages 6 to 57 years) from Copenhagen University Hospital and Lund University Hospital (see Table 1. for patient table). Post-surgicals evaluation of the hippocampus were performed by a pathologist at respective hospitals, and diagnosis for hippocampal sclerosis was determined according to ILAE guidelines[17]. The use of resected patient tissue and following procedures were approved by the local Ethical Committee in Copenhagen (H-2-2011-104) and Lund (#212/2007) and were performed in accordance with the Declaration of Helsinki. Written informed consent was obtained from all subjects prior to each surgery.

**Acute slice preparation.** The hippocampal tissue is surgically removed *en bloc* and then cut in the coronal plane once or twice to establish orientation of the hippocampal structures and aid in the positioning of the tissue when slicing. The resected tissue is then placed in ice-cold sucrose solution, frozen to slush, containing (in mM): 200 sucrose, 21 NaHCO3, 10 glucose, 3 KCl, 1.25 NaH2PO4, 1.6 CaCl2, 2 MgCl2, 2 MgSO4 (all from Sigma-Aldrich, Sweden), adjusted to 300–310 mOsm, 7.4 pH. The tissue is either transported from Copenhagen University Hospital, Rigshospitalet to Lund (60–90 min) or between the surgery room at Lund University Hospital and the electrophysiology lab in the neighbouring building (15 min). Depending on the orientation of the hippocampus, the tissue is either trimmed to give a better surface to glue on, or glued straight away onto the cutting platform. The 400 µm thick, coronal slices are cut with a vibratome (VT1200, Leica Microsystems) in ice-cold sucrose solution, continuously bubbled with 95% $O_2$ and 5% $CO_2$.

**Acute slice incubation.** Slices were collected in a pre-incubation bath with aCSF, containing (in mM): 129 NaCl, 21 $NaHCO_3$, 10 glucose, 3 KCl, 1.25 $NaH_2PO_4$, 2 $MgSO_4$, and 1.6 $CaCl_2$, adjusted to 300–310 mOsm, 7.4 pH, heated to 34 °C and continuously bubbled with carbogen (95% $O_2$ and 5% $CO_2$). Slices rested on nets, fully submerged, for 15–30 min before they were transferred to the interface incubation chamber. This incubation consisted of a closed chamber with humidified air inside created by a bubble stone continuously bubbling carbogen into aCSF covering the bottom of the closed chamber. Inside this chamber, an open box with a constant flow of carbogenated aCSF was placed containing the slice holder, with six holes for cell culture insets (Millipore, Germany), positioned in the box to just touch the surface of the circulating aCSF (Fig. 1). The slices were placed onto the cell culture membranes of the insets, resulting in constant aCSF flow below the membrane and humidified air from above. The membrane enabled slices to access aCSF (with nutrients and defined ion composition)

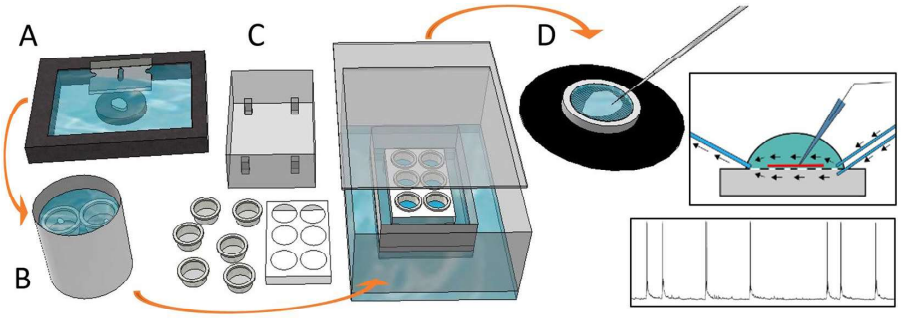


**Figure 1.** Overview of Experimental setup. (**A**) The tissue is resected *en bloc* and sliced on a vibratome. (**B**) The slices rest for 15 min submerged in continuously bubbled (95% $O_2$/5% $CO_2$) aCSF and (**C**) are then moved to the incubation chamber for 3–48 hours of incubation with a constant flow of bubbled aCSF in the small chamber and humidified air crated by bubbling the aCSF in the outer box which is sealed with a lid. After incubation the slices are transferred to the dual-flow recording chamber (**D**) where electrophysiological whole-cell patch-clamp recordings were made.

while the humidified air gave maximised access to oxygen, preventing drying of slices. Care was taken to minimize bacterial growth, especially for slices incubated for 48 hours, and the aCSF in circulation was changed every 12 hours to prevent accumulation of bacteria. During the course of experiments, a UV-C light was added to the circulation tubing to eliminate bacteria in the aCSF. It was installed in the recirculation loop of the aCSF, outside of the incubation box, and great care was taken to not illuminate anything else except the circulating aCSF. The slices rested in the interface chamber, at room temperature between 3 and 48 hours.

The study was based predominantly on the analysis of both structural and functional characterization of dentate gyrus of the hippocampus, since this was the area most commonly identified to be less damaged and present in all tissue pieces that we received from the surgical resections of the temporal lobe.

**Electrophysiology.** Electrophysiological recordings were performed with glass capillary electrodes (tip resistance between 2.5 and 6 MΩ) backfilled with a solution containing in mM: 122.5 K-gluconate, 12.5 KCl, 10 KOH-HEPES, 0.2 KOH-EGTA, 2 Mg-ATP, 0.3 Na3GTP, and 8 NaCl, pH 7.2–7.4 (mOsm 290–300) with HEKA amplifier (HEKA, Germany) controlled with HEKA Patchmaster software. Visualisation of the cells was achieved by an Olympus microscope BX51WI (Olympus, Germany). The slices were individually transferred to the dual flow-recording chamber and held in place by a horseshoe-shaped piece of platinum wire. The slice was perfused with carbogenated aCSF, preheated to 32 °C, at a flow rate of 2 ml/min. The slice rested on a metal grid allowing for a laminar flow of aCSF directed above and beneath the slice, ensuring a high amount of oxygen available for the slice and enabling the use of a submerged objective for infrared differential contrast (IR-DIC)-imaging, giving visual cues for patch-clamp recordings. Visual cues for whole-cell patch clamp excluded neurons with a dark distinguishable nucleus or a swollen or shrunken soma. After successful giga-seal formation, the patch was ruptured and the resting membrane potential (RMP) was immediately measured in current-clamp recording mode before continuing with other recording sequences. Experiments with an access resistance over 30 MΩ were excluded from analysis. For field-recordings, capillaries with a tip resistance between 1 and 3 MΩ, backfilled with aCSF were used.

**Incubation and induction of epileptiform activity.** Slices were transferred for recordings, starting after 3 hours to allow for recovery of dendritic spines that retract in the ice-cold cutting solution, as reported in preparations of acute rodent hippocampal slices[5]. Electrophysiology recordings were obtained from slices incubated from 3 to 48 hours. The initial RMP-measurement was followed by depolarising current steps (stepwise increasing with 50 pA/step) to determine AP-threshold and firing pattern. Spontaneous activity of neurons was recorded in current-clamp mode for at least 15 min. Two different types of excitability-enhancing aCSF-solutions were used, either [0$Mg^{2+}$]-aCSF alone, with magnesium omitted, or [0$Mg^{2+}$]-aCSF containing the potassium-channel blocker 4-AP (100 µM). Following recording, the slices were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) overnight and stored submerged in Walter's antifreeze solution (ethylene glycol and glycerol in PBS) at −20 °C.

Immunohistochemistry To produce tissue samples suitable for immunohistochemical staining, slices were removed from the antifreeze solution and washed three times in KPBS, embedded in a solution, containing 300 g/L egg-albumin (Sigma) and 30 g/L gelatin (Sigma) in Milli-Q water, and stored at −20 °C. Further sectioning was performed using a cryostat (Cellab Nordia AB) to produce 20 µm coronal sections. These were mounted on slides (+charged Menzel-Glas, Thermo Scientific) and stored at −18 °C before staining. Slices were rinsed three times with KPBS and pre-incubated for 1 hour at room temperature in blocking solution, consisting of 10% normal donkey serum (NDS) in 0.25% Triton X-100 in KPBS (T-KPBS). After blocking, slices were incubated in darkness at 4 °C with the appropriate dilution of primary antibody in 5% NDS in T-KPBS (see Suppl. Table 1) overnight. After rinsing three times in T-KPBS, they were incubated for 2 hours in darkness at room temperature with secondary antibody (1:200) in 5% NDS in T-KPBS (see Suppl. Table 1), after which they were rinsed

three times in T-KPBS. The procedure was then repeated for the second staining. After the secondary antibody was applied, the slides were rinsed once in T-KPBS and twice in KPBS. Finally, slides were coverslipped with DABCO (Sigma-Aldrich D2522). In the Iba1 + GFAP-stainings, Hoechst 33342 (Hoechst AG) diluted to 1:1000 in DABCO was applied. Images were taken using an Olympus BX61 microscope (Olympus, Germany) fitted with a CCD camera connected to a Windows PC with cellSens Dimension software (Olympus, Germany). For NeuN + CASP3- and GFAP + Iba1-stainings, images overviewing the dentate gyrus and an area surrounding the tip of the dentate gyrus were taken, respectively, at 20× magnification. For NeuN + NPY-stainings, images were taken overviewing the dentate gyrus and the hilus at 10× magnification.

**Statistics and Analysis.** Analysis of electrophysiology recordings was performed with IGOR Pro (Version 6.3, Wavemetrics) and Mini analysis software (Synaptosoft). Postsynaptic potentials (PSPs) were automatically detected with a detection threshold of 1 mV (Synaptosoft) and subsequently checked manually to eliminate double peaks during a 60 s timeframe. To further analyse the PSP properties, the first 12 PSPs from the five cells with the highest PSP frequency in each group were added together resulting in 60 PSPs from each of the time points: 3, 24 and 48 hours respectively. The parameters of PSPs and APs were detected and analysed automatically by Mini analysis software (Synaptosoft), with the duration measured as the width at half-amplitude. The resting membrane potential was calculated by an average of data points from the first 100 ms from current clamp recording start. The distribution of data was tested with D'Agostino & Pearson normality test as well as plotted according to frequency in histogram to evaluate if the data were normally distributed before further statistical analysis in Prism software (Graphpad 7). Normally distributed data were analysed with one-way ANOVA, while for data detected as not normally distributed, the Kruskal-Wallis test was used (Prism software, Graphpad 7). Analyses of immunohistochemical data were done using the Fiji/ImageJ software (https://imagej.nih.gov/). The area of the dentate gyrus was measured as the area between the innermost and outermost granule cells along the entire length of the structure, from which NeuN-positive and CASP3-positive cells were individually counted. Values for NeuN-positive cells/mm$^2$, CASP3-positive cells/mm$^2$ and CASP3-positive cells/NeuN-positive cells were calculated for each slice. NPY-positive cells/mm$^2$ were calculated from NPY-positive interneurons in the hilus. The hilus was delineated by the border between granular cell layer and the hilus and a straight line drawn between the end-points of dentate granule cell layer against CA3/4 area. Iba1-assessment was performed in an hilus area, adjacent to the tip of the dentate gyrus, measuring 5mm$^2$, in which all cells were counted and assessed by as Ramified (inactive; small soma with fine cellular processes), Intermediate (bigger, elongated soma with thicker proximal processes) or Activated (round or amoeboid cells with few or short processes) microglia. Cells that could not be assessed as a specific state were also included when counting overall cell density. Values were calculated for Ramified cells, Intermediate cells, Activated cells, as well as total Iba1-positive cells/mm$^2$. GFAP was assessed with optical densiometry (Image J[18,19]) using an area of 1mm$^2$ in the hilus adjacent to the tip of the dentate gyrus, extracting values for minimum, maximum and mean grey values and integrated optical density. Statistical analyses were performed using SPSS Statistics 24 (IBM) and data from the 3, 24 and 48-hour time points in each patient were normalized against 0 h. Normality of data was examined using Shapiro-Wilk's test of normality with normally-distributed data evaluated using repeated-measures ANOVA with post-hoc Bonferroni correction and non-normally distributed data assessed using Friedman's test.

## Results

**No detectable changes in neuronal morphology or apoptosis in slices after 48 hours of incubation.** The viability of human hippocampal slices incubated in our interface system was first examined by assessing several immunohistochemical markers at 0, 3, 24 and 48 hours. We chose to focus our entire analysis on dentate gyrus because it was always present and most preserved part of the hippocampus after the resection. Microtubule-associated protein 2 (MAP2) immunostaining showed clear and abundant neuronal morphologies in slices from both 0 and the 48-hour time point (Fig. 2A). A closer examination of the dentate granule cell layer did not reveal any differences in neuronal morphology between slices incubated for 0, 3, 24 or 48 h (Fig. 2B). To evaluate potential changes in neuronal density over time, we stained for the neuronal nuclear marker, NeuN, and counted number of cells in the granule cell layer of the dentate gyrus. Cell density analysis of NeuN-positive granule cells showed variability between patients but no signs of significant changes over time in any of the tissue obtained from individual patients (Fig. 3B and Supplementary Table 2). Consistent with maintained morphological features, counterstaining for apoptotic marker Cas3, did not show any increase in expression across the time-points studied (Fig. 3C and Supplementary Table 2). We also found the same number of cells positive for both neuropeptide-Y (NPY) and NeuN throughout the incubation, indicating that also NPY-expressing interneurons were viable over time (Fig. 3D and Supplementary Fig. S1 and Supplementary Table 2). Finally, microglial- and astrocytic activation was assessed in the slices. Microglial cells, labelled by Iba1 were counted and different cellular states (Ramified, Intermediate and Activated, respectively) were analysed in a 5 mm$^2$ area in the hilus, adjacent to the tip of the dentate gyrus. A clear decrease in Ramified microglial cell numbers was observed from 0 h to 3 hours and further to 24 hours ($45 \pm 3.1$ to 4.75, $n = 3$, $p = 0.0131$, paired *t-test*). Optical density measurements for GFAP-immunostaining within an area of 1 mm$^2$ at the tip of the hilus of the dentate gyrus did not show any trend for change over the time of incubation (Supplementary Table S2) in any of the parameters measured. Taken together, these results suggest that there are neither detectable changes in morphology or overall density of neurons, nor astrocytes in the slices from different time points, although microglia activation increase over time in incubation.

**Intrinsic neuronal properties after 3, 24 and 48 hours of incubation.** As neuronal density and overall morphology of neurons seemed to be unchanged after 48 h of incubation, we proceeded to confirm these

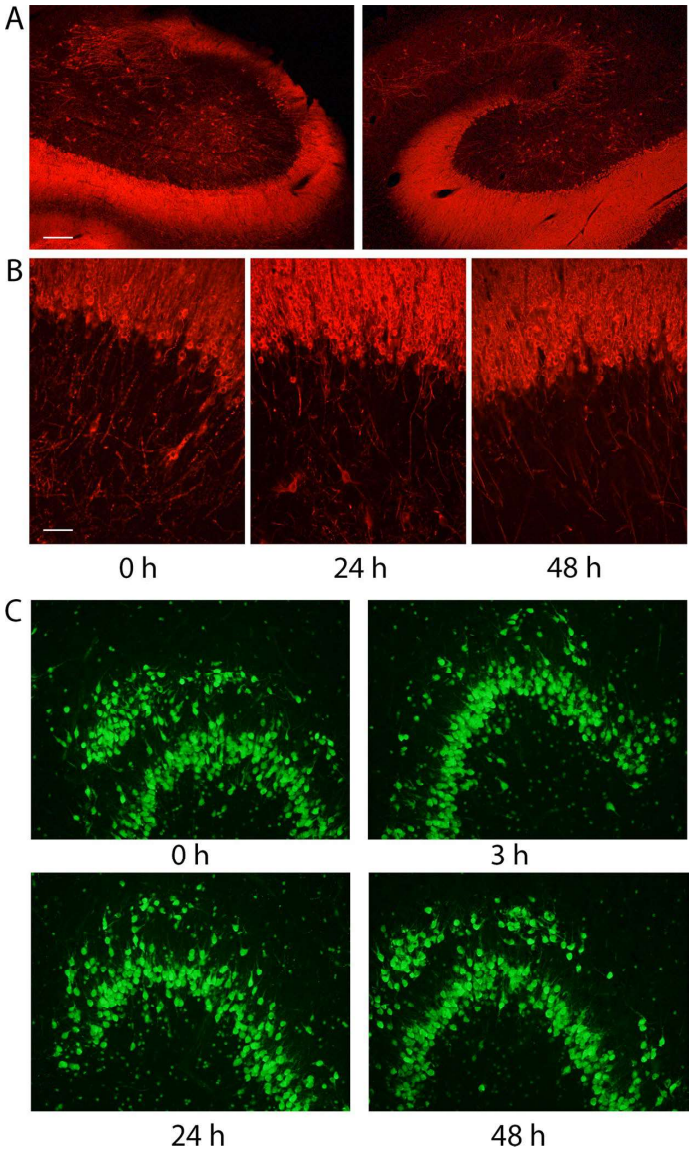


**Figure 2.** Interface incubation of human hippocampus maintain hippocampal organisation. (**A**) Hippocampal slices incubated for 0 and 48 hours from the same patient (patient number 12, no hippocampal sclerosis) stained for MAP-2 in red did not show any structural changes between time-points, (**B**) neither did the granule cells in the dentate gyrus differ between 0, 24 and 48 h from the same patient. (**C**) Staining for neuronal nuclear marker, NeuN in green, did not show any morphological differences between time-points in a patient diagnosed with hippocampal sclerosis (patient number 15). Scale bars 20 µm.

morphological findings by assessing functionality of the neurons, starting by investigating their intrinsic properties at three time-points of the incubation period. Whole-cell patch-clamp recordings from dentate granule cells in the double-flow chamber were performed after interface chamber incubation for 3, 24 or 48 hours. The total number of granule cells recorded at the different time intervals were 16, 34 and 19 for 3, 24 and 48 hours, respectively. The RMP of all cells were estimated immediately after breaking the membrane for whole-cell recordings. A small but statistically significant reduction in average RMP in the 48 h group was detected (Fig. 4A, Table 2). The average RMP at 48 hours was $66.47 \pm 1.27$ mV, while at 3 and 24 hours RMP was $70.62 \pm 1.36$ and $70.04 \pm 0.74$ mV, respectively. Tukey's test of multiple comparisons ($n = 69$, $p = 0.0219$, ANOVA) identified a significant difference between the 48 hours group and the 3 and 24 hours group (3 hours, $70.62 \pm 1.36$ mV, $n = 16$, compared to 48 hours,

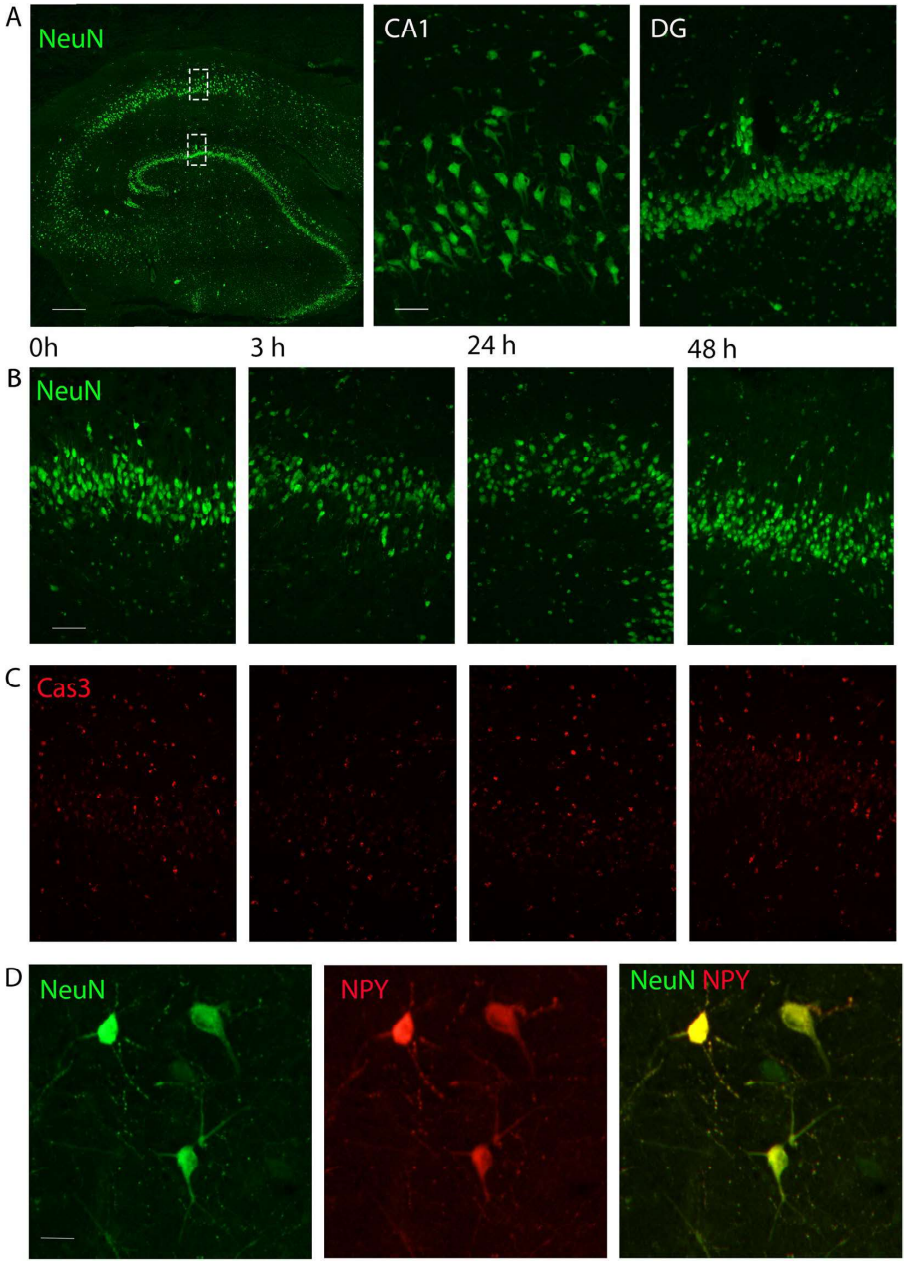


**Figure 3.** Human hippocampus shows no difference in the number of neurons, expression of apoptotic marker or NPY-interneuron number in interface incubation. (**A**) Neuronal nuclear marker, NeuN in green (scalebar 500 μm), outlining cell layers magnified to the right for CA1 and dentate gyrus (scalebar 100 μm). (**B**) No differences were found in number of NeuN-positive neurons in the dentate granular layer over the time studied (green, scalebar 100 μm, Patient 16), (**C**) nor in the number of neurons positive for the apoptotic marker Cas3 (red). (**D**) Example of NPY-expressing interneurons positive for NeuN (green) and NPY (red) found in the hilar region of dentate gyrus from 48 h time-point. Scalebar 20 μm.

66.47 ± 1.27 mV, $n = 19$, p = 0,0414, 24 hours, 70.04 ± 0.74 mV, $n = 34$ compared to 48 h, 66.47 ± 1.27 mV, $n = 19$, p = 0.0372). No statistically significant differences between the 3 hour and 24 hour groups were detected (3 hours, 70.62 ± 1.36 mV, $n = 16$, compared to 24 hours, 70.04 ± 0.74 mV, $n = 34$, p = 0.9198). Granule cells displayed APs

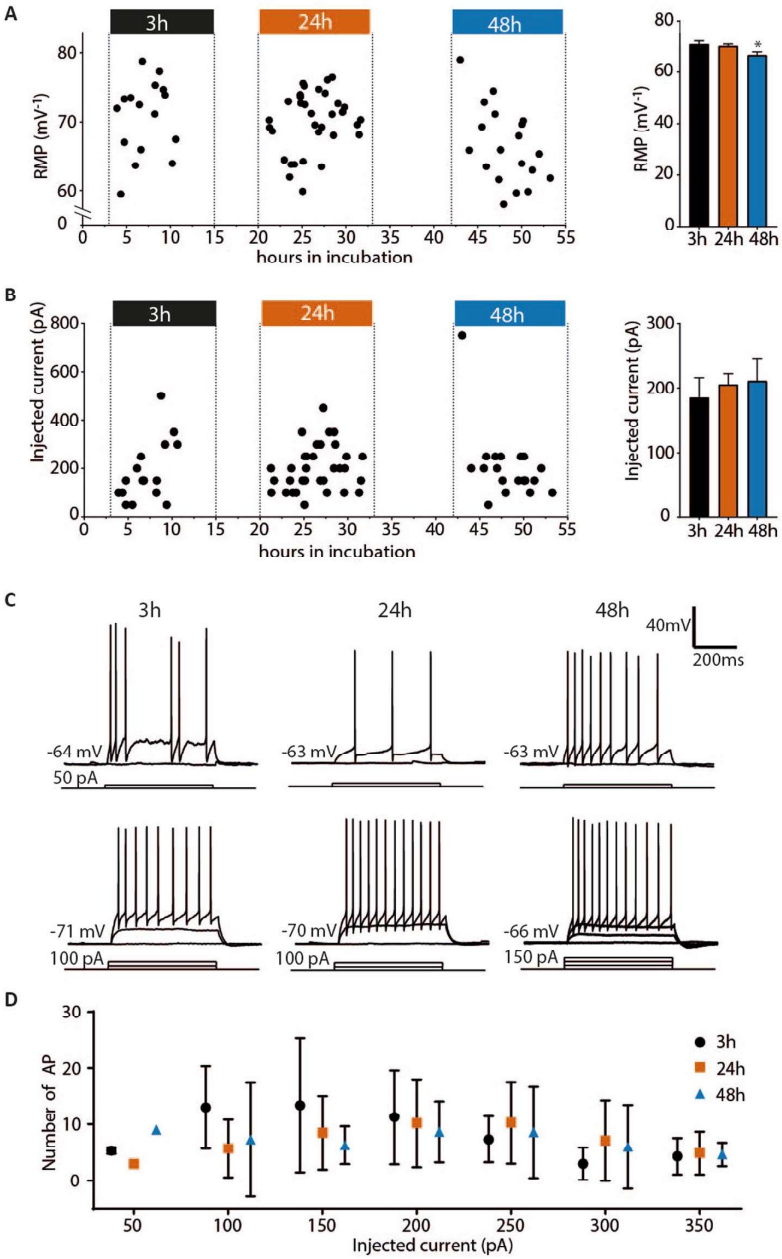


**Figure 4.** Intrinsic properties of human dentate granular cells. (**A**) The left graph show each cell, represented as a dot, with RMP on the y-axis and number of hours in incubation on the x-axis. Dotted lines and coloured blocks indicate the three time-groups (3 h, 24 h and 48 h). A small but significant change was detected in the RMP for the 48 hour group ($n = 69$, $p = 0.0219$, ANOVA) illustrated in the bar graph to the right (Tukey's multiple comparison test: 3 hours, $70.62 \pm 1.36$ mV, $n = 16$, compared to 48 hours, $66.47 \pm 1.27$ mV, $n = 19$, $p = 0{,}0414$, 24 hours, $70.04 \pm 0.74$ mV, $n = 34$ compared to 48 h, $66.47 \pm 1.27$ mV, $n = 19$, $p = 0.0372$). No statistically significant differences between the 3 hour and 24 hour groups were detected (Tukey's multiple comparison test: 3 hours, $70.62 \pm 1.36$ mV, $n = 16$, compared to 24 hours, $70.04 \pm 0.74$ mV, $n = 34$, $p = 0.9198$). (**B**) The left graph show the lowest current injection step (50 pA/step) needed to generate AP for each cell, represented by a dot, with amount of current injected on the y-axis and number of hours in incubation on the x-axis. The right graph illustrate the lowest current injection step needed to generate AP for cells binned to 3, 24 and 48 hours, with no differences between the three groups ($n = 69$, Kruskal-Wallis test, $p = 0.5481$). For Input

resistance, AP threshold, AP amplitude and AP half-width with each cell represented by a dot and number of hours in incubation on the x-axis see Supplementary Fig. S1. (**C**), upper panel, example recordings of the lowest current injection step (50 pA/step) needed to generate AP in cells incubated for 3, 24 and 48 hours. Lower panel, example recordings of the highest number of AP generated during a depolarisation step from cells incubated for 3, 24 and 48 hours. (**D**) Number of AP plotted at each depolarisation step (50 pA/step) from 50 to 350 pA with mean number of AP and SEM for each group (3, 24 and 48 hours).

upon stepwise depolarisation and no changes were detected in the amount of injected current needed to trigger an AP (Fig. 4B, Table 2) ($n = 69$, Kruskal-Wallis test, $p = 0.5481$). The number of AP for each depolarisation step (50 pA/step) at the three incubation time points is illustrated in Fig. 4D with example recordings of the lowest depolarisation step and the highest number of AP for each of the three time points. The input resistance and AP properties were recorded from each cell with no changes detected between the three groups (Mean and SEM in Table 2, individual cell values plotted against time and p-numbers in Supplementary Fig. S2).

**Spontaneous postsynaptic currents after 3, 24 and 48 hours of incubation.** To investigate if the pattern of synaptic inputs to neurons was altered in slices over time in incubation, we recorded and analysed spontaneous PSPs in dentate granule neurons in slices at 3, 24 and 48 hours of incubation (Fig. 5, Table 3). The frequencies of PSP events sampled during the first 60 seconds of recording were not statistically different between the three groups (3 hours incubation: $0.23 \pm 0.08$ Hz $n = 16$, 24 hours incubation: $0.29 \pm 0.06$ Hz $n = 34$ and 48 hours incubation: $0.17 \pm 0.05$ Hz mV $n = 19$, Kruskal-Wallis test, $p = 0.2307$). No differences were detected in any parameter of PSPs analysed, including amplitude (3, 24 and 48 hours incubation, $n = 60$ in each group, Kruskal-Wallis test, $p = 0.4391$), half-width (3, 24 and 48 hours incubation, $n = 60$ in each group, Kruskal-Wallis test, $p = 0.9528$), rise time (3, 24 and 48 hours incubation, $n = 60$ in each group, Kruskal-Wallis test, $p = 0.4583$) or decay time (3, 24 and 48 hours incubation, $n = 60$ in each group, Kruskal-Wallis test, $p = 0.1921$). We also verified that no subtle change in PSP amplitude could be detected between the three groups by generating cumulative probability curves (Fig. 5C). No shift in the PSP amplitude cumulative probability curves could be detected (comparing 3 and 24 hours incubation, $n = 60$ in each group, Kolmogorov-Smirnov test, $p = 0.3752$; comparing 3 and 48 hours incubation, $n = 60$ in each group, Kolmogorov-Smirnov test, $p = 0.1813$; comparing 24 and 48 hours incubation, $n = 60$ in each group, Kolmogorov-Smirnov test, $p = 0.3752$), indicating maintained network connectivity over the entire time of incubation. Taken together, these data suggest that intrinsic electrophysiological properties and afferent synaptic inputs to dentate granule cells during the 48-hour incubation period are maintained without major alterations.

**Spontaneous and evoked epileptiform activity after 3, 24 and 48 hours of incubation.** To be able to use our platform as an *in vitro* validation tool for new antiepileptic treatments in human pharmacoresistant epileptic tissue, we investigated if dentate granule cells would display spontaneous or induced epileptiform activity at different time-points of incubation. We observed spontaneous epileptiform activity manifested as bursting AP activity in four dentate granule cells during perfusion with normal aCSF (Fig. 6A,B): three cells from one slice incubated for 3 hours, and one cell from a slice of a different patient incubated for 48 hours. Although the pattern of epileptiform activity of the cells from these two patients was somewhat different, the short burst-like epileptiform activity observed in the slice incubated for 3 hours was similar in all three cells (example, Fig. 6B). The epileptiform activity appeared in trains of AP bursts, each train lasting between 12 and 29 s, with the number of bursts during each train ranging from 10 to 16, and the number of APs in each burst from two to five APs. The frequency of the APs during one burst spanned from 43 Hz up to 294 Hz. The time interval between each burst train ranged from 3 to 11 min, one cell having more regular time interval between bursts (mean time of $6.00 \pm 0.37$ min). The spontaneous epileptiform activity (bursting) was abolished when NMDA (D-AP5) or AMPA (NBQX) receptor antagonist were applied to the perfusion medium (Supplementary Fig. S3). In the second patient tissue where spontaneous epileptiform activity was observed (Fig. 6A), the cell displayed one SLE with a high frequency AP onset (200 Hz), terminating with three distinct bursts.

The low rate and irregularity of spontaneous epileptiform events in normal aCSF makes this approach inadequate to evaluate and validate a potential therapeutic effect of novel treatment strategies. Therefore, to evoke more regular and higher frequency epileptiform events, we tested 3 different protocols that have been previously used for this purpose: (i) [$0Mg^{2-}$]-aCSF alone, or in combination with (ii) electrical stimulation or (iii) potassium channel blocker 4-AP. Reducing magnesium-block of the NMDA receptor by [$0Mg^{2+}$]-aCSF has been shown to readily induce seizure-like activity in human hippocampal slices[20]. In our study, application of [$0Mg^{2+}$]-aCSF gave rise to SLEs in three out of six recorded dentate granule cells from 6 slices (Fig. 5C), with a high frequency onset of APs of more than 150 Hz, followed by sporadic bursts with AP frequency of more than 50 Hz. Combining [$0Mg^{2+}$]-aCSF with electrical stimulation, similar to stimulus-induced bursting (STIB), induced SLEs with similar burst frequency (in 2 dentate granule cells from 2 slices, Supplementary Fig. S4). Finally, with the addition of the potassium channel blocker 4-AP to the [$0Mg^{2+}$]-aCSF, we observed robust and regular bursting in 6 out of 11 dentate granule cells recorded (example Fig. 5D–F). The time interval between the bursts ranged from about 1 s to 41 s, with burst duration ranging from 16 to 630 ms. Number of APs during a burst varied from 2 (the minimum number of APs to be defined as a burst) to a maximum of 36, with an average of 6.9 APs per burst. Firing frequency of APs during the bursts was on average 54 Hz, ranging between 8 Hz and 264 Hz. In one cell, apart from the bursts, a SLEs with high frequency AP onset (180 Hz) and with 3 to 10 terminating bursts was also observed. Another cell displayed bursting of APs in trains with a duration between 10 and 13 s. The number of bursts during each train ranged from 16 to 22, and the number of APs in each burst ranged from 2 to 17. The

| | 3 h ($n = 16$) | 24 h ($n = 34$) | 48 h ($n = 19$) |
|---|---|---|---|
| RPM (mV) | −70.62 ± 1.36 | −70.04 ± 0.74 | −66.47 ± 1.27 |
| Input R (MΩ) | 185.50 ± 30.53 | 170.40 ± 60.83 | 164.7 ± 21.55 |
| AP threshold (mV) | −43.05 ± 1.50 | −43.82 ± 0.90 | −42.87 ± 1.12 |
| AP half-width (ms) | 0.65 ± 0.03 | 0.63 ± 0.02 | 0.63 ± 0.03 |
| AP amplitude (mV) | 96.85 ± 3.04 | 99.27 ± 1.51 | 94.8 ± 2.01 |

**Table 2.** Intrinsic properties.

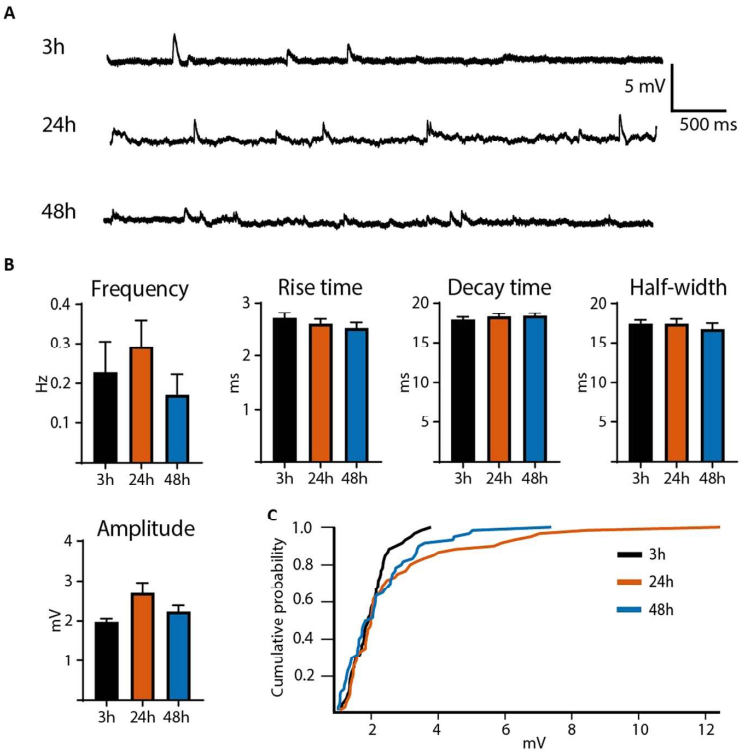


**Figure 5.** No difference in PSP properties was observed between 3, 24 and 48 hours of incubation. (**A**) Representative baseline recordings with normal aCSF showing EPSPs at 3, 24 and 48 hours of incubation. (**C**) No difference was detected when measuring frequency (3 h, $n = 16$, 24 h incubation, $n = 34$ and 48 h incubation, $n = 19$, Kruskal-Wallis test, $p = 0.2307$), amplitude ($n = 60$, Kruskal-Wallis test, $p = 0.4391$), half-width ($n = 60$, Kruskal-Wallis test, $p = 0.9528$), rise time ($n = 60$, Kruskal-Wallis test, $p = 0.4583$) or decay time ($n = 60$, Kruskal-Wallis test, $p = 0.1921$) of the PSPs generated by cells from slices incubated for 3, 24 and 48 hours. (**C**) PSP amplitude for each group (3, 24 and 48 hours) plotted as cumulative probability show no difference between the three groups (cumulative plot of PSP amplitude: comparing 3 and 24 hours incubation, $n = 60$ in each group, Kolmogorov-Smirnov test, $p = 0.3752$; comparing 3 and 48 hours incubation, $n = 60$ in each group, Kolmogorov-Smirnov test, $p = 0.1813$; comparing 24 and 48 hours incubation, $n = 60$ in each group, Kolmogorov-Smirnov test, $p = 0.3752$).

first burst in each train displayed similar characteristics as an SLE, with a high frequency onset and longer duration, but was lacking the terminating bursts. The evoked epileptiform activity was reduced in the presence of the NMDA receptor antagonist D-AP5, further decreased by adding AMPA receptor antagonist NBQX, and was fully abolished in the presence of NMDA, AMPA and GABA (PTX) receptor antagonists (See supplementary Fig. S5). This suggests that the epileptiform activity evoked by [$0Mg^{2+}$]/4-AP-aCSF is dependent on both glutamatergic and GABAergic receptors. Further, simultaneous paired field and whole-cell recordings from the molecular layer and a granule cell showed recurrent, epileptiform activity in field recordings, synchronised to burst activity in the whole-cell recording (See supplementary Fig. S6). Applying our incubation protocol to human resected tissue from a cortical dysplasia patient demonstrated both viable cells and normal electrophysiological properties at

| | 3 h ($n=60$) | 24 h ($n=60$) | 48 h ($n=60$) |
|---|---|---|---|
| Amplitude (mV) | 1.98 ± 0.08 | 2.70 ± 0.26 | 2.23 ± 0.16 |
| Half-width (ms) | 17.41 ± 0.46 | 17.42 ± 0.57 | 16.75 ± 0.73 |
| Rise time (ms) | 2.72 ± 0.10 | 2.61 ± 0.09 | 2.53 ± 0.11 |
| Decay time (ms) | 17.91 ± 0.34 | 18.32 ± 0.38 | 18.43 ± 0.32 |

**Table 3.** Spontaneous synaptic potentials.

24 hours time point, with an average RMP of −69.2 ± 3.1 mV, and AP amplitude 93.4 ± 6.7 mV (n = 5), as well as robust rhythmic epileptiform bursts in response to [$0Mg^{2+}$]/4-AP-aCSF (See supplementary Fig. S7), indicating that maintained neuronal properties over the first 24-hours is not dependent on the selective resilience of DG neurons to stress, but is rather due to the used incubation procedure. Further studies are needed, however, to confirm viability of cortical neurons over 48-hours.

## Discussion

Here we present a method for human brain slice incubation where human hippocampal slices are viable and functionally preserved for 48 hours after resection from patients with drug-resistant temporal lobe epilepsy. We provide evidence that this unique tissue could be used for electrophysiological experiments for a prolonged period, therefore enabling increased number of experiments to be carried out from the same resection surgery, as well as allowing for broader experimental approaches, such as fast-expressing viral vector based gene therapy strategy validations. Moreover, we present data demonstrating that the dual flow-recording chamber represents an unprecedented opportunity for visually guided whole-cell patch-clamp recording of individual neurons, and at the same time enabling induction of seizure-like activity in human hippocampal tissue slices. We also report that whole-cell recordings from human dentate granule cells reveal robust seizure-like activity in epileptic dentate gyrus up to 48 hours of incubation. Interestingly, *spontaneous* epileptiform activity in the epileptic dentate gyrus, an area where no such activity has been previously reported, was occasionally observed in normal aCSF. The presented method significantly expands the time span for recordings in acute human brain slices, substantially increasing the window of opportunity for electrophysiological recordings, and thereby maximising the period for broad electrophysiological data acquisition when using this unique human brain tissue.

We found that incubating human hippocampal slices under interface conditions for 48 hours did not lead to a decrease in number of neurons in the dentate granule layer (Fig. 2, Supplementary Table 2), nor did it reduce the number of NPY-expressing interneurons (Fig. 3, Supplementary Table 2), suggesting good survival capacity of the slices under these conditions. We detected, however, an increase in the number of activated microglia after 48 hours (Supplementary Table 2). When activated, microglial cells start expressing pro-inflammatory cytokines, such as interleukins, TNF-α and IFN-γ, to recruit neighbouring microglia and trigger broader inflammatory processes around them[21]. Activated microglia are also known for their ability to modulate neuronal circuitry in their vicinity, either by removing presynaptic terminals ("synaptic stripping"[22,23]) or regulating synaptic receptors through signalling molecules[23], for example by up-regulation of postsynaptic AMPA-receptor expression through signalling via TNF-α[22]. Present data in human slices, however, do not indicate that such changes occur in synaptic transmission, at least in the dentate gyrus, since our electrophysiological experiments show no significant changes in PSP-properties at 48 h: neither amplitude, duration, rise time nor decay time of PSPs were altered. No significant differences were detected in the frequency of spontaneous postsynaptic events, indicating that synaptic connectivity, as measured by PSPs, did not change over the 48 hours of incubation. The RMP of dentate granule cells was slightly lower at this time-point (for about 3 mV on average) although other intrinsic membrane properties or AP firing properties were not different between 3, 24 and 48 hours of incubation (Fig. 4, Table 2), indicating that the cells have functionally intact membrane, with ion pumps and channels, capable of maintaining RMP and generation of normal APs. RMP values for human dentate gyrus cells has previously reported to be -62.9 ± 1.24 mV[24], which is in agreement with our data.

Spontaneous rhythmic burst activity with clear paroxysmal depolarisation shifts (PDS) was observed, in normal aCSF, in four dentate granule cells (three with AP during the PDS and one without the AP) in one slice after 3 hours of interface incubation. To our knowledge this is the first time spontaneous epileptiform burst activity has been recorded in the dentate gyrus of human epileptic hippocampal slices[25,26]. Previously, Richard Miles' group showed spontaneous activity, recorded in the subiculum and the CA2 area of the human hippocampus[13,27]. The burst activity recorded in the dentate gyrus by us is very similar in shape and frequency to the activity previously recorded from pyramidal cells in the subiculum[13]. The major difference is that this activity seen in the dentate gyrus came in burst-trains with several minutes of silence in between the bursts, while previously observed activity from subiculum was continuous rhythmic burst activity without long intervals. The common consensus on describing characteristics for SLEs is a fast onset followed by irregular spiking, ending with periodic bursting[28]. This matches with our spontaneous recordings: an onset of AP frequency over 150 Hz followed by attenuated AP frequency that stabilizes for a few seconds and then terminates by three clearly defined bursts. In general, SLE activity (assessed by field recordings in temporal neocortex) has been demonstrated previously exclusively in the interface recording chamber[14], but has not been observed using the submerged-recording chamber as shown here. Our findings support the conclusion that the submerged dual-flow perfusion chamber presumably allows for comparable conditions, e.g., oxygen levels, to the interface-recording chamber, without compromising visual access for whole-cell patch-clamp recordings as is the case for the interface-recording chamber.

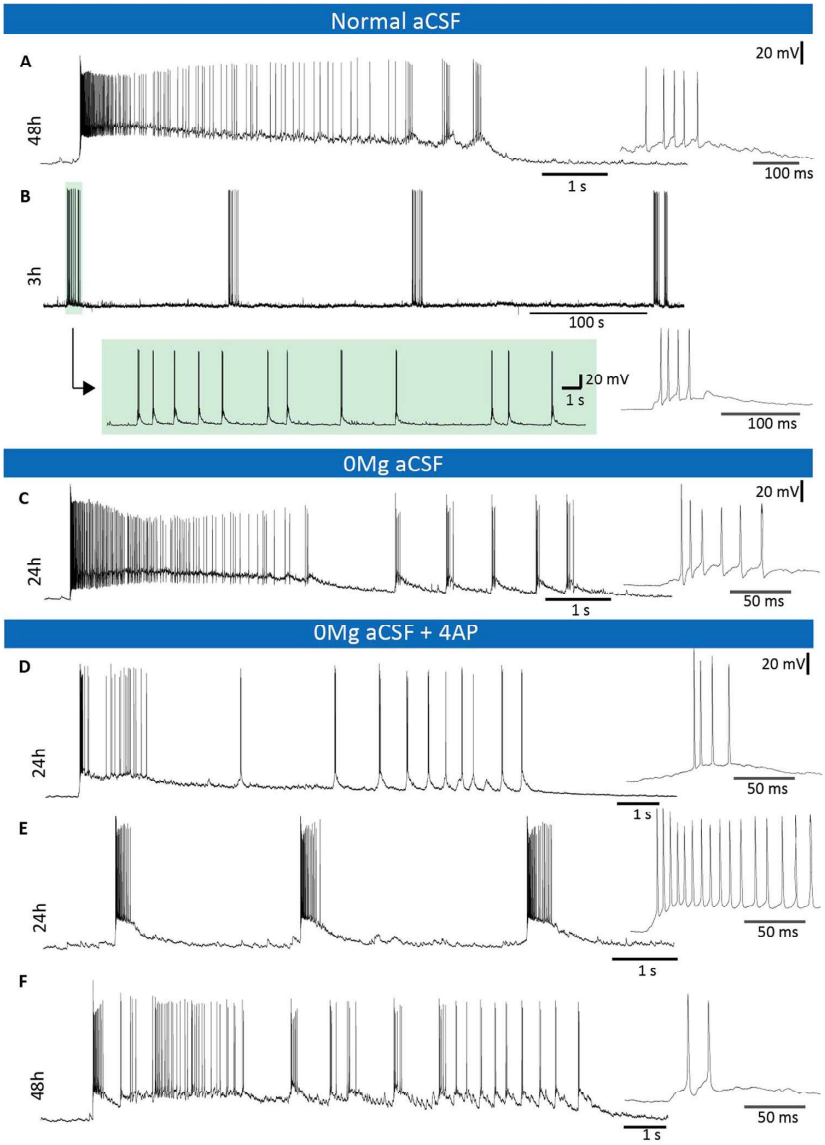


**Figure 6.** Spontaneous and evoked SLEs and epileptic burst activity observed after 3, 24 and 48 hours of incubation. (**A**) Spontaneous SLE was observed in normal aCSF after 48 hours of incubation, with a high frequency onset of 200 Hz and three terminating bursts. (**B**) Epileptiform burst activity recorded after 3 hours of incubation with 12–25 s long burst-trains every 3–11 min with 10–16 bursts in each train. The bursts consisted of up to five APs and each burst-train lasted 12–29 s. Between each burst the membrane potential returns to baseline until the onset of the next burst. (**C**) In slices incubated for 24 hours, SLEs with more than 150 Hz firing frequency at the onset and approximately 60 Hz in the following bursts were evoked by excluding $Mg^{2+}$- from the aCSF. (**D**) By adding 4-AP to the [$0Mg^{2-}$]-aCSF solution the activity became regular, with 150 Hz at the onset and around 40 Hz during the following bursts. (**E**) Example of regular bursts from a cell while perfused by the [$0Mg^{2+}$]-aCSF solution with 4-AP added. (**F**) After 48 h of incubation it was still possible to evoke SLEs under perfusion of the [$0Mg^{2+}$]-aCSF solution with 4-AP added.

Epileptiform activity in the dentate gyrus (as judged by whole-cell patch-clamp recordings from granule cells) was successfully evoked by both [$0Mg^{2+}$]-aCSF as well as [$0Mg^{2+}$]/4-AP-aCSF. In cells perfused with [$0Mg^{2+}$]-aCSF, we observed SLEs with similar features to the spontaneous SLE (Fig. 5). The recurrence of these SLEs was highly variable between the recorded cells, ranging from a single SLE to one every 8 minutes. The slices

perfused with [0$Mg^{2+}$]/4-AP-aCSF rarely displayed SLEs (1 in 10 cells), but instead exhibited strong AP bursts in 5 out of 10 cells (Fig. 5D–F). The epileptiform activity evoked by [0$Mg^{2-}$]/4-AP-aCSF were comparable to the spontaneous bursts in aCSF. It is clear that both [0$Mg^{2+}$]-aCSF and [0$Mg^{2-}$]/4-AP-aCSF can generate epileptiform activity as revealed by recordings obtained from the dentate granule cells in the double-flow chamber. The [0$Mg^{2+}$]-aCSF caused SLEs very similar to the one that occurred spontaneously during normal aCSF perfusion.

To be able to test new compounds and treatments, however, robust and continuous epileptiform activity is required. The slices perfused with [0$Mg^{2+}$]/4-AP-aCSF generate such robust activity, with bursts that exhibit similarities to those that follow spontaneous SLEs seen under perfusion with normal aCSF, making this protocol suitable for evaluating novel treatments for suppression of seizure-like events in human epileptic tissue.

Taken together, the method presented here with long incubation time and ability to generate robust epileptiform activity is a favourable platform for pre-screening of novel therapeutic approaches, particularly those that require longer observation times (e.g. to allow for viral vector-based gene expression), as a validation step in patient-derived pharmacoresistant epileptic tissue before proceeding to clinical trials. In addition, the double-flow chamber makes it possible to induce seizure-like events with a visual approach for whole-cell recordings readily available. The latter may increase the sensitivity of the platform, as well as deepen our understanding of how individual neurons contribute to epileptiform activity in human pharmacoresistant epileptic tissue.

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## Author Contributions

J.W., M.K. and M.A. wrote the main text, J.W., M.A., N.G.B. and R.V. performed experiments. L.H.P. and B.J. performed all surgery. All authors contributed to experimental design and reviewed the manuscript.

## Additional Information

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# Paper IV