



# Optogenetic kindling

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## **Abstract**

Epilepsy is a common disease that affects about 1 % of the population. The neurological disease is characterised by episodes of neural hyperactivity (seizures) and neural synchronization that can have behavioural manifestations such as convulsions. Anti-epileptic drugs (AEDs) can be used for treating most cases of epilepsy, but since especially temporal lobe epilepsy (TLE) has proven to be drug resistant, there is still a need to develop novel treatment methods. The research towards such new treatments requires animal models that mimic the pathology of the human disorder. In this project, optogenetics is used in evoking seizure-like afterdischarges (ADs) in anaesthetised mice by stimulating the CA1 region of the hippocampus. The mice are transgenic, expressing channelrhodopsin 2 – green fluorescent protein (ChR2-GFP) under the Thy1 promoter. An optrode is constructed and used to both stimulate with 463 nm light in 10 Hz pulses and to record an intracranial electroencephalogram (EEG). The tissue response and amount of ADs that follows a light stimulation train is evaluated. Also, spontaneous activity is assessed by calculating the burst suppression rate (BSR). Isoflurane and ketamine anaesthesia is compared and it is concluded that ketamine at the dose of 80mg/kg blocks the development of ADs following a stimulation train. On the other hand, the isoflurane anaesthetised animals developed ADs which seemed to escalate in a kindling like manner.

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## Sammanfattning på svenska

Epilepsi är en vanlig neurologisk sjukdom som drabbar ungefär 1 % av befolkningen. Beroende på vilken del i hjärnan som är påverkad så blir manifestationerna av ett anfall olika. Om hyperaktiviteten är lokal och inte sprider sig kan patienten bli frånvarande och få automatismer. Generaliserade anfall är mer allvarliga och medför ofta stora krampanfall. En mycket vanlig form av epilepsi är temporallobsepilepsi (TLE). Denna typ utgår oftast ifrån hippocampus och tenderar att vara motståndskraftig mot medicinering. Om fallet är allvarligt övervägs ett kirurgiskt ingrepp där det sjuka området avlägsnas, men detta måste noga vägas mot de konsekvenser som en sådan lesion kan innebära. Eftersom behandlingen för TLE idag är bristfällig så finns det ett behov av att utveckla nya behandlingsmetoder, något som både kräver grundforskning kring epilepsi och innovation. Hjärnan är ett mycket komplext organ med multipla celltyper som är kopplade i ett intrikat nätverk. Det är idag svårt att återskapa ett ekvivalent system, och därför genomförs forskning i intakt hjärnvävnad. Majoriteten av experimenten är dock för farliga för att utsätta människor för, och då det saknas alternativ måste försöken göras på djur.

En djurmodell härrar i något avseende patologin från den mänskliga sjukdomen. Detta examensarbete är ämnat att bidra till arbetet med att ta fram en optogenetisk krampmodell. Optogenetik är ett relativt nytt verktyg för neurologisk forskning och ger forskare möjlighet att påverka specifika nervcellpopulationer med en hög tidsupplösning. Tekniken bygger på att man med hjälp av genteknik för in ljuskänsliga protein, opsin, i cellmembranet i vissa specifika nervceller. De aktiveras av ett begränsat spektrum av ljus och ändrar flödet av joner genom cellmembranet. Effekten på nervcellen varierar beroende på vilken typ av opsin som införts. Opsin kan antingen depolarisera eller hyperpolarisera cellmembranet, vilket leder till en initiering eller en inhibering av aktionspotentialer. I detta projekt har det depolariserande opsinet Channelrhodopsin 2 (ChR2) använts för att skapa hyperaktivitet i hippocampus i genmodifierade möss. Ett stimuleringsprotokoll med upprepade ljuspulser användes för att provocera fram epileptisk aktivitet i form av efterurladdningar (afterdischarges).

Djuren är sövda under experimenten, och eftersom sövningsmedlen i viss utsträckning interagerar med samma cellulära mekanismer som opsinen gör, så finns det anledning att tro att det finns en interaktion. Målet med detta examensarbete är att utröna vilka effekter valet av narkosmedel har på den optogenetiska krampmodellen. För att undersöka detta jämfördes två grupper av möss; den ena sövd med inhalationsmedlet isoflurane, den andra med det injicerade ketamine/xylazine. För att komplettera undersökningen fick även ett djur en falsk stimulering under isoflurane-narkos.

Efter experimenten utvärderas mängden efterurladdningar och spontan aktivitet. Vissa nya analysmetoder fick tas fram för att tackla variabilitet och störningar i EEG inspelningen, bl.a. med hjälp av matematiska analysprogram och filterfunktioner. Av resultaten kan man dra slutsatsen att ketamine, i den dos som användes i dessa experiment, är ett olämpligt narkosmedel för den optogenetiska krampmodellen. Djuren i denna grupp utvecklade inte urladdningar i samma utsträckning som de som var sövda med isoflurane. Dessutom så uppkom en mycket kraftig störning i EEG inspelningen i ketamindjuren, som kunde härledas till djurens andning. Denna störning överskuggade mer eller mindre den intressanta neurala aktiviteten. De djur som var sövda med isoflurane uppvisade dock efterurladdningar. Dessa verkade dessutom eskalera under experimentets gång, något som skiljer sig från vad som tidigare rapporterats.

## Abbreviations

ANN	Artificial neural network
AEDs	Anti-epileptic drugs
BMC	Biomedical centre
BSR	Burst suppression rate
CA	Cornus ammonis
CamKII	Ca <sup>2+</sup> /calmodulin-dependent protein kinases II
ChR2	Channelrhodopsin 2
CNS	Central nervous system
EEG	Electroencephalogram
EPSP	Excitatory post synaptic potential
GABA	γ-aminobutyric acid
GFP	Green fluorescent protein
IBE	International bureau of epilepsy
ILAE	International league against epilepsy
i.p.	intra peritoneal
IPSP	Inhibitory post synaptic potential
NpHR	Natronomonas Pharaonis Halorhodopsin
PV	Parvalbumin
RMS	Root mean square
SEM	Standard error of the mean
TLE	Temporal lobe epilepsy
XFP	X fluorescent protein

# 1. Introduction

Epilepsy is a severe disease which can cripple the life of the sufferer, never knowing when a seizure may strike. The seizures are often exhausting for the patient and are in many cases dangerous to both health and the surroundings. As of today there are many anti-epileptic drugs (AEDs) available, although with some significant side effects, such as drowsiness and nausea. Some of the patients respond to the pharmacological treatment and can expect to be seizure free. Though, there are some types of epilepsy that are more resistant to drugs than others. One of those types, temporal lobe epilepsy (TLE), which is emerging from the hippocampus, is the most common form of epilepsy in humans [1].

There are few alternatives to the pharmacological treatment approach. An invasive surgical operation removing the focal centre is one, but sometimes the consequences of an operation, such as loss of speech or hearing, are not acceptable. There is therefore a need to develop novel methods to engage the problem. The development of such new treatments will require studies of the disease *per se* and evaluation of new innovations.

In order to study complex diseases, such as epilepsy, scientists are utilizing animal models which serve to mimic the nature of the disorder. These models express one or many features of a disease which can be studied in acute or chronic experiments. In epilepsy studies, a frequently used model is the “electrical kindling model” where a rat brain is focally stimulated with an intracranial electrode giving repetitive pulses until seizures emerge. As this procedure is repeated, the duration of the following seizures increase gradually and the behavioural signs are also elevated. It is argued that this model share many features with TLE, if the kindling is performed in the hippocampus [2].

This master’s thesis is a part of a project where optogenetics is utilized to refine the TLE kindling model. Optogenetics is based on bioengineered ion channels (opsins), originally found in various unicellular organisms, which can be expressed in brain cells using different gene techniques. Once they are activated by light, they change their permeability to specific ions. The change in permeability will result in a net flux of ions that will either hyper- or depolarize the cell. This technique is capable of activating or deactivating specific areas and cells in the brain, by exciting or inhibiting neurons [3]. Massive activation by exciting neurons is believed to have similar effects on the brain as the electrical kindling method has. Previous studies have shown that a train of light pulses will be followed by afterdischarges alike those pursuing an electrical kindling protocol [4].

One major challenge in using optogenetics *in-vivo* is delivering the light to the tissue and simultaneously recording the signals from this stimulated volume. One approach to tackle this is to use an optrode, which is an optic fibre fused with a recording electrode. This probe can be inserted into the area of interest, in this case the hippocampus, through the skull bone; a procedure that requires that the animal is put under anaesthesia.

Previous work with the optogenetic seizure model in this laboratory has resulted in a hypothesis that anaesthetic drugs interact negatively with the development of afterdischarges. Some anaesthetics target the same cellular functions as the optogenetics and it is postulated that there might be interference. Since the occurrence of afterdischarges in the model is crucial for future TLE research applications, it is necessary to maximize the abundance of these disease mimicking features. Therefore, the anaesthetic effects on the optogenetic seizure model must be explored.

By exchanging the previously used anaesthetic isoflurane to a combination of ketamine and xylazine, which has a different mechanism of action, we expect to see more afterdischarges developing in the animals.

## **2. Aims of the thesis**

This master's thesis primary objective is to contribute to the work of refining and optimizing the optogenetic seizure model, in order to make it an effective tool in epilepsy research. The three specific aims with this work are:

- To characterize the optogenetic seizure model, in order to establish if it is possible to predict the occurrence of afterdischarges and other electrographic events.
- To explore whether and how the anaesthetic drug isoflurane affects the optogenetic seizure model.
- To optimize the procedure in respect to anaesthetic method and optrode position.

### 3. Theory

This section contains background information that is necessary for understanding the experimental procedures and the discussion of the results. Some topics are not used in the methodology, but are addressed because they are crucial for the future translation into clinical applications.

#### 3.1 Epilepsy

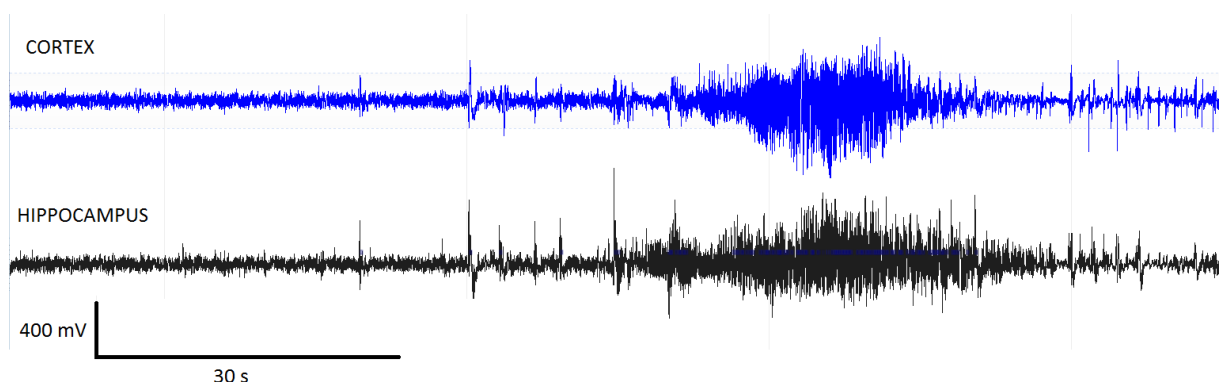
The most used definition of epilepsy is based on a consensus between the international league against epilepsy (ILAE) and the international bureau of epilepsy (IBE). It reads as follows:

*“An epileptic seizure is a transient occurrence of signs and/or symptoms due to abnormal excessive or synchronous neuronal activity in the brain [5].”*

*“Epilepsy is a disorder of the brain characterized by an enduring predisposition to generate epileptic seizures and by the neurobiologic, cognitive, psychological, and social consequences of this condition. The definition of epilepsy requires the occurrence of at least one epileptic seizure [5]. ”*

The massive synchronous activity in the brain during a seizure can be detected when recording an electroencephalogram (EEG) (see figure 1). Electrodes are placed on specific locations on the skull bone and measure the potentials that arise from the neuronal signalling. When more detailed observations are necessary, as in many research applications, the electrodes can be placed intracranial directly in the tissue of interest.

A seizure (ictus) often causes loss of awareness and can be accompanied with behavioural manifestations that cause severe exhaustion, such as convulsions. In some cases, the seizure is preceded by an aura, where for example a distinct feeling of fear or a strange abdominal sensation tells the patient that a seizure is near [6]. The generalized tonic-clonic seizure (grand-mal) is the most infamous form of seizure where the patient suffers from massive convulsions. There are also less violent forms, such as absence seizures (petit mal) where the patients lose consciousness, that never the less disturb the everyday life of the sufferer. In many cases, the sufferers and their relatives are also experiencing the indirect effects of the disease such as prohibition to drive vehicles, fear of new seizures and incomprehension from employers [7, 8].



*Figure 1. Intracranial EEG recording from cortex and the hippocampus during an epileptic seizure in a rat.*



Over 40 diseases sort under the above mentioned definitions, all of them classified in respect to origin and clinical manifestations [9]. Two main classes of seizures constitute the basis of the classification. *Generalized epileptic seizures* spread rapidly in a bilateral network involving a large part of the brain but not necessarily the entire cortex. The onset point and seizure propagation may vary from time to time. The other category, the *partial epileptic seizures*, has the same point of origin which spreads the hyperactive signalling along preferred paths. In some cases secondary generalization occur where the epileptiform activity spreads to the whole brain [6, 10].

In the year of 2011, 6301 persons in Sweden were diagnosed with epilepsy in closed care [11], and it is estimated that a total of 60000 individuals suffer from the disease [6]. Prevalence in other countries varies between 3- 11 / 1000 [12].

The most common form of epilepsy is the TLE which originates from the mesial temporal limbic structures [1]. Even though the precise location of origin is debated there are signs that indicate that the hippocampus is closely related to the disease [13]. The seizures of TLE are primarily partial complex seizures which often develop into secondary generalizations [1]. These typically have three components; an aura, disturbed consciousness and automatisms. The latter means more or less uncontrolled motoric activity i.e. lip smacking or belly scratching. As the composition among the components varies, the seizure characteristics differ between patients [6]. TLE is often accompanied by hippocampal sclerosis and brings a bad prognosis of the patient being seizure free because of its resistance to AEDs [1].

The treatment of epilepsy is dependent on type and severity. Pharmacological compounds, AEDs, are the most subscribed and focus on altering the ion transport over the cell membrane, thereby affecting the excitability of the neurons. By targeting ion channels such as  $\text{Na}^+$ -channels and  $\text{GABA}_A$  it is possible to dampen the hyperactivity that causes seizures by enhancing the inhibitory mechanisms in the brain [14].

Surgical methods are evaluated, if AEDs are not efficient, to conclude whether a focal centre can be removed without undesirable effects. In severe and very rare cases, the brain hemispheres are separated by cutting the corpus callosum to restrict the spreading of the seizure [15]. Although this particular operation is extreme, smaller surgical methods are suggested to be utilized more frequently because of the good prognosis of becoming seizure free in some types of epilepsies [1].

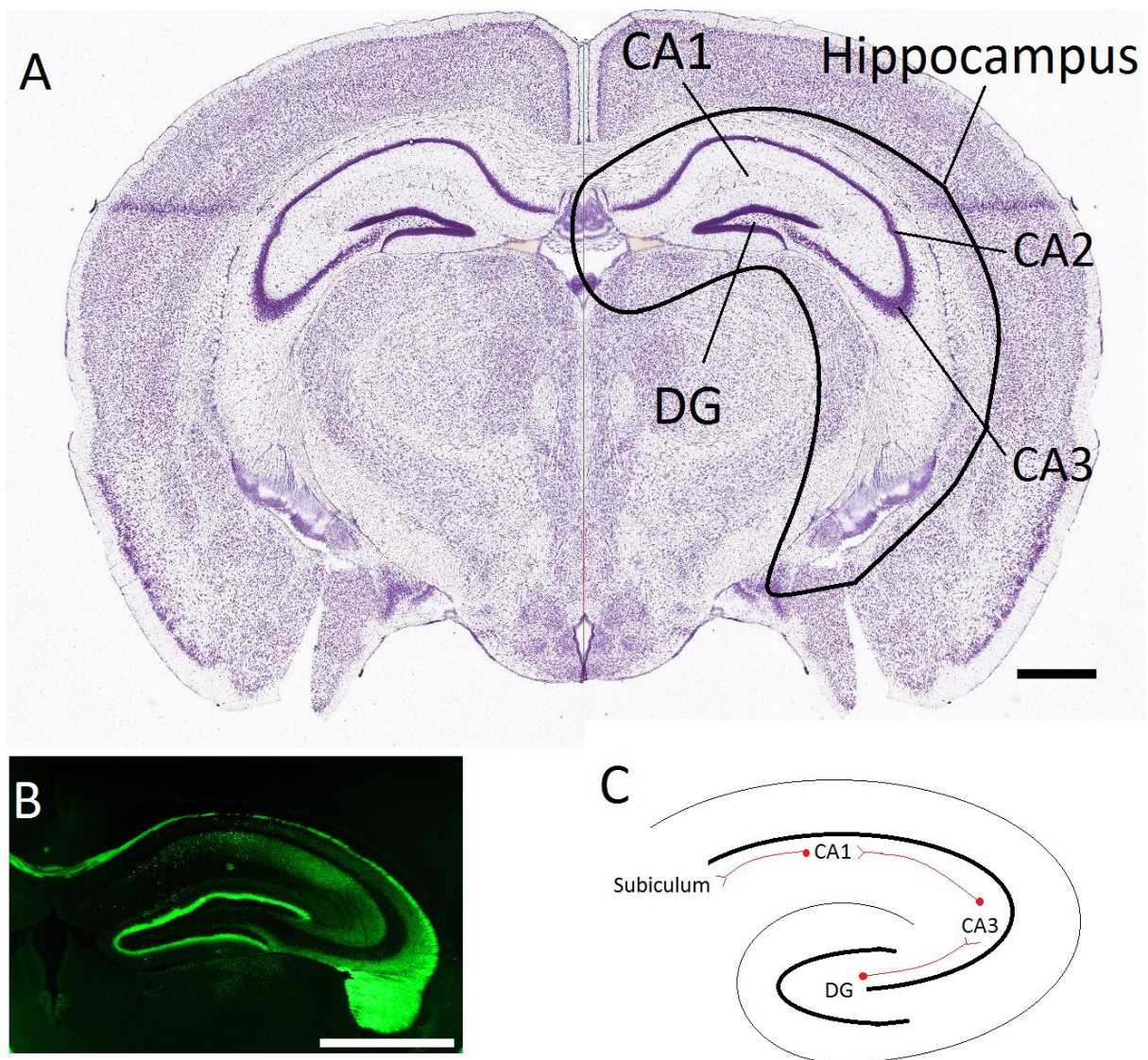
### 3.1.1 Hippocampus

The hippocampal formation is located in the temporal lobe and is composed of a couple of substructures, one of them being the hippocampus. These structures are considered to be involved in the facilitation of memories and spatial orientation [16]. The anatomy of the hippocampus differs from other cortical neural structures because of its mostly unidirectional signalling pathways [17]. Excitatory inputs enter the entorhinal cortex where it is transmitted to the dentate gyrus through the perforant pathway. The axons of the granule cells in the dentate gyrus, called mossy fibres, are then connected to pyramidal cells in the CA3 region via the hilus. CA derives from Cornus Ammonis, the horn of the Egyptian god Ammon. CA3 cells signal to the CA1 region by the Schaffer collaterals. The CA1 projects to the subiculum via the alveus and the pyramidal cells of the subiculum connect again to the deep layers of the entorhinal cortex. These connections involving the CA1, CA3 and dentate gyrus is called the feed-forward tri-synaptic circuit [18] (see figure 2).

There are mainly two types of neurons in the hippocampus, pyramidal cells and interneurons. The pyramidal cells are in majority and use glutamate as neurotransmitter while the interneurons release  $\gamma$ -aminobutyric acid (GABA). These two cell types serve as counterparts; pyramidal cells are

excitatory and evoke excitatory post synaptic potentials (EPSP) in target neurons while interneurons inhibit activity by inducing inhibitory post synaptic potentials (IPSP) in target neurons [19]. An EPSP will depolarise the post synaptic membrane and, if strong enough, initiate an action potential. The IPSP, on the other hand, will hyperpolarise the membrane preventing action potentials to initiate [16].

Some argue that the underlying cause of TLE is an imbalance between these inhibitory and excitatory mechanisms. However, the opinions of what causes this imbalance differ. One group of scientists claim that GABAergic inhibition in fact synchronise the neural network into massive epileptiform activity [20]. Another view is that lack of GABAergic inhibition sometimes makes the system unable to prevent seizures [21].



*Figure 2. (A) Coronal Nissl stained slice of a mouse brain. The hippocampus, with the dentate gyrus (DG) and cornus ammonis 1 (CA1) region, is marked. Adopted from Allen Institute of Science in accordance with their citation policies [22]. Scale bar: 1 mm. (B) Chr2-GFP expression in hippocampus. Intense green correlates to high expression. Scale bar: 1 mm. (C) Schematic picture of the feed-forward tri-synaptic circuit in the hippocampus.*

## 3.2 Optogenetics

One of the inventors of optogenetics (together with Edward Boyden) Karl Deisseroth defines the method as:

*“Optogenetics is the combination of optical and genetic methods to achieve gain or loss in function of well-defined events in specific cells of living tissue [3].”*

The method, which is based on membrane bound light responsive proteins called opsins, has developed steadily since invented and was first implemented in neurons in 2005 [23]. Even though the technique as such is new, it has been desired for a long time. As early as 1979, the Nobel laureate Francis Crick predicted that there would be a need to inactivate one specific cell type without altering the functions of others in order to fully interrogate the complexity of the brain [24]. In addition to such specificity, a method to interrogate the circuits of the brain should have fast kinetics to provide high temporal control of the neural activity.

Classical neurological methods to manipulate neurons such as electrical stimulation, pharmacological compounds and genetic techniques have not yet been able to combine both the cell type specificity and the fast kinetics required to exert such precise control of neural circuits. Electrical stimulation with electrodes provides the high temporal resolution that is needed to excite or inhibit single action potentials in neurons. Though, even if the time resolution is adequate, it is hard to achieve specific control of one cell type because of the general electrical influence the electrodes have on their immediate surroundings. In the other end, pharmacological methods and genetic approaches are able to target specific cell types, but have too slow kinetics to control discrete spiking in neurons [25]. Optogenetic methods combine the fast elements from the electric stimulation with the high selectivity that is achieved with gene techniques and provide a new approach to interrogate and control the brain (see table 1).

Since optogenetics has proven to be such a powerful tool, it is suggested to be recruited to many areas of neurological research. It is for example suggested to be utilized in moderating epilepsy by exciting interneurons in the hippocampus [26].

*Table 1: Optogenetic stimulation methods combine high cell specificity with fast kinetics.*

	ELECTRIC	CHEMICAL / GENETIC	OPTOGENETIC
KINETICS	Fast	Slow	Fast
SPECIFICITY	Low	High	High

### 3.2.1 Opsins

The opsins belong to the protein family retinylidene which use retinal as a cofactor. They are divided into two subgroups depending on their origin. The archaeal type (Type I) is expressed mainly in prokaryotes where they function as ion pumps or contribute to phototaxis. Type II are found in higher eukaryotes and work as photoreceptors in eyes [27]. Even though the two groups show remarkable similarities they are not phylogenetically related and have developed independently of each other [28].

The first opsin was found in a microbe called *Halobacterium Halobium* in 1971 [29]. The protein (bacteriorhodopsin) proved to be a proton pump that was activated by light [30]. To this date multiple different opsins have been discovered. Their common feature is that they are 7-transmembrane proteins that form a pocket in which the vitamin A derived chromophore retinal is covalently bound [31]. When retinal is bound to the opsin the complex is called rhodopsin and their light associated functions are utilized in many organisms, both prokaryotic and eukaryotic. Retinal (by chance) proved to be abundant in vertebrate central nervous system (CNS) neurons and is not considered to be lacking in optogenetic applications [32].

It is the cofactor retinal that reacts to light by isomerization. The type I opsins make use of the all-*trans* retinal change to 13-*cis* retinal upon photon interaction (see figure 3). The rhodopsin complex then initiates its photocycle which ends with the thermal re-isomerization of the retinal back to its ground state [33]. The wavelength which activates the rhodopsin is dependent on the charges surrounding the retinal Schiff base (RSB) linkage to the opsin [27].

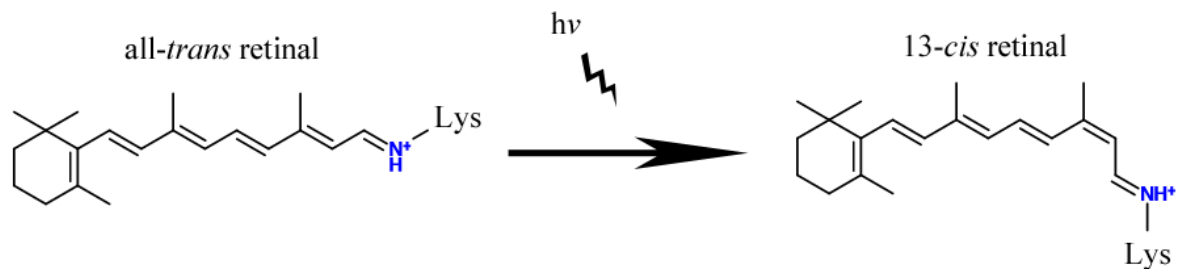


Figure 3: The light induced isomerization of all-*trans* retinal to 13-*cis* retinal. In this illustration, retinal is bound to an opsin by the lysine (Lys) linkage.

To this date there are numerous opsins available that have different activation light wavelengths and functionality. Most of them function as light activated ion channels or pumps that either hyperpolarize or depolarize the cell membrane, but recently G-protein coupled functionality has been developed as well (OptoXR) [34].

The most commonly used opsins are Bacteriorhodopsin (BR), Halorhodopsin and the Channelrhodopsin 2 (ChR2). These three membrane bound proteins change the membrane permeability of different ions and hence affect the membrane potential in cells. BR is a light driven proton pump that is suggested to harvest light energy by building a pH gradient over the membrane [30, 31]. ChR2 is a non-selective cation ( $H^+$ ,  $Ca^{2+}$ ,  $Na^+$ ,  $K^+$ ) channel and will depolarize the membrane upon activation. Another type of opsins is hyperpolarising; the Halorhodopsin that is derived from the *Natronomonas Pharaonis* (NpHR) can selectively pump  $Cl^-$  into the cytosol preventing action potentials to form [35] (see figure 4). It is suggested that this type of opsins could be used in suppressing epileptic seizures [36].

ChR2, derived from the alga *Chlamydomonas reinhardtii*, is often used when working with excitatory optogenetics. This opsin is activated by blue-green light (about 470 nm) and is very fast acting. There is roughly a 6 ms latency between light onset to peak amplitude in hippocampal neurons [37, 38]. ChR2 is the opsin of choice for the optogenetic seizure model.

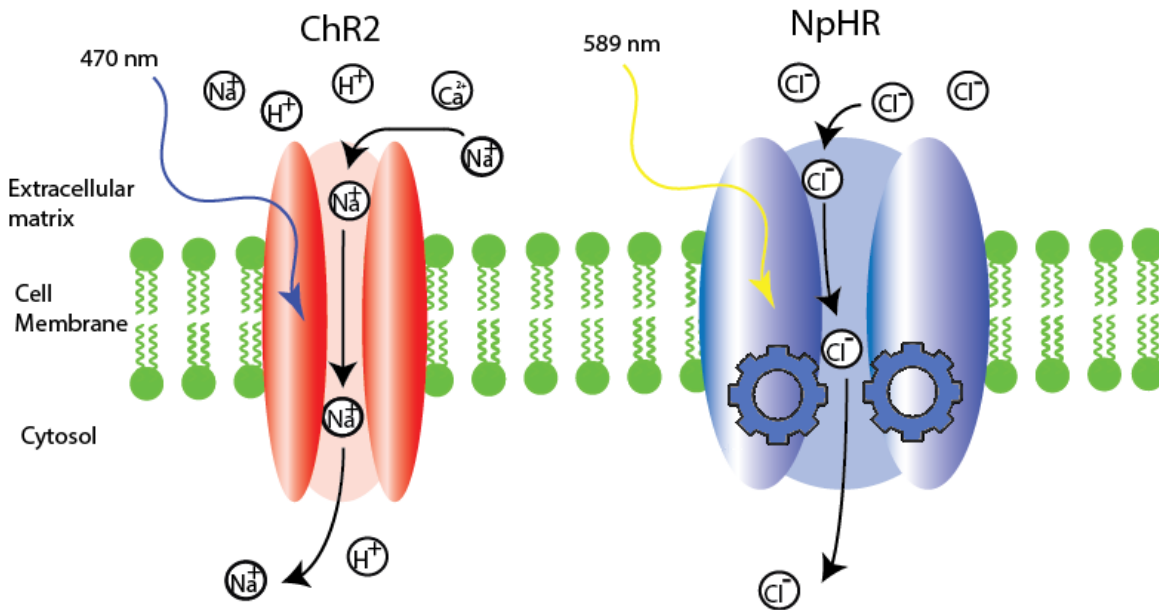


Figure 4: Schematic illustration of ChR2 and NpHR in the cell membrane. ChR2 is permeable to cations and NpHR selectively pumps chloride ions upon light interaction. The two types of opsins react to different wavelengths of light.

### 3.2.2 The membrane potential

All living cells depend on the ionic environment both inside and outside the cell to stay alive. Regulating the ionic concentrations in the cytosol in respect to the extracellular environment is a fundamental and energy consuming task that is required for the maintenance of homeostasis. There are multiple types of proteins in the membrane dedicated to the task of controlling the transport of ions in and out of the cell. These ion channels can actively (by the consumption of ATP) or passively allow passage of specific ion types across the membrane. The specific permeability for each ion is dependent on the selectivity of the ion channels. Two forces will act on the ions; diffusion along their respective concentration gradient and electric forces due to their charge. These forces combine into the electrochemical driving force. If one type of ion is actively transported into the cell, the result will be a concentration gradient but also a separation of charge and a resulting electric potential across the membrane. This membrane potential is typically around -60 to -70 mV at resting state in neurons.

The Goldman equation describes the equilibrium potential  $E_m$  over the membrane.

$$E_m = \frac{RT}{F} \ln \frac{P_K[K^+]_{out} + P_{Na}[Na^+]_{out} + P_{Cl}[Cl^-]_{out}}{P_K[K^+]_{in} + P_{Na}[Na^+]_{in} + P_{Cl}[Cl^-]_{in}}$$

This equation considers concentration of each ion [X] as well as the permeability  $P$  and also the temperature  $T$ , the ideal gas constant  $R$  and the Faradays constant  $F$ . If divalent ions such as calcium is included, the equation gets a bit more complicated [39], but in the case of the resting membrane potential calcium is of little significance due to the low permeability.

The ions of interest are mainly Na<sup>+</sup>, K<sup>+</sup>, and Cl<sup>-</sup>, each having special roles to play in the functions of the neuron. The opsins in optogenetics momentarily change the permeability for one or a set of ions upon activation. This will result in a flow of ions across the membrane and hence a shift in membrane



potential. Generally, an increase in cation permeability will result in a depolarisation and an increase in  $\text{Cl}^-$  permeability will hyperpolarise the cell [16].

### 3.3 Genetic techniques

The opsin is introduced to the cells of interest by gene techniques. Transgenic animals (the majority being mouse models) which are expressing opsins in certain brain areas are available, supplying scientists with a flexible tool for various experiments [35]. A transgenic animal is generated by e.g. pronuclear injection where fertilized mice eggs are injected with DNA coding for a specific protein [40].

Another option is to deliver the genes *in-vivo* using viral vectors. This method is crucial for future optogenetic clinical applications. Virus vectors such as lentivirus (LV) and adeno-associated virus (AAV) have been used for introducing both excitatory and inhibitory opsins to *in-vivo* tissue [25]. The virus vector is injected in the brain area of interest and subsequently infects a large portion of the cells in the vicinity of the injection. The specificity is achieved by using genetic promoters known to be expressed by the target cell type alone.

One of the most promising virus vectors for gene therapy in neurological diseases is the AAV [41]. It is capable of invading both dividing and non-dividing cells, which is of essence since most cells of interest for neurological applications are in a post mitotic state. AAV also achieves robust expression for long periods of time without pathogenicity [41-43]. The possibility to manufacture large amounts of purified vectors without contamination of wild type AAV also adds to the suitability of using AAVs in clinical applications [44].

A typical transgene involves a promoter that regulates the expression, a functional gene and a reporter. GFP is a commonly used reporter, that will help to confirm the expression during or after the experiment (see figure 5).



Figure 5: Schematic illustration of a simple genetic construct of an AAV vector. The Inverted Terminal Repeat (ITR) sequences related to the AAV are flanking the construct. A promoter serves to regulate the expression, followed by the opsin coupled to a reporter.

#### 3.3.1 Promoters

Gene expression is regulated by genetic sequences preceding the gene that codes for a protein. These parts are called promoters and work as locks for expressing proteins. To unlock the gene, transcription factors need to bind to the promoter. Then the transcription into mRNA can begin. What specifies a cell type is its sets of transcription factors that regulates the gene expression, since the entire genome is present in each cell. The amount of expression is regulated by the number of transcription factors dedicated to one promoter [45].

The promoters are used to target a specific cell type with gene therapy. There are neuronal populations that alone express one specific type of protein. The promoter of such protein can then be used preceding a transgene in a vector. Expression of this transgene will then be directed to only the cell type of interest even though the virus infects all cells.

There are numerous known promoters that can be used to target different types of neurons. For example, the  $\text{Ca}^{2+}$  / calmodulin-dependent protein kinase II  $\alpha$  (CaMKII  $\alpha$ ) has been used to target

excitatory pyramidal neurons [46] and the Parvalbumin (PV) promoter can be used to specify the expression to a certain class of interneurons [47].

### **3.3.2 The Thy1 promoter**

Another promoter considered to target neurons is the Thy1 promoter. It regulates the Thy1 protein which is an immunoglobulin expressed on the surface of projection neurons and thymocytes [48]. The use of the promoter results in random expression in neurons in general which varies depending on the transgenic line [49]. One research group reported Thy1 driven XFP (X Fluorescent Protein) expression in hippocampal pyramidal cells in transgenic mouse lines [48]. These lines were created by injecting fertilized oocytes with gel purified DNA. Based on these findings the Thy1 promoter was successfully utilized to drive expression of ChR2-GFP in transgenic mice for optogenetic applications [50]. Expression was found to be prominent in many areas of the brain, including the hippocampal CA1 and CA3 region [37]. These mice were founders to the ones used in this project.

## **3.4 Animals**

Some experiments that are necessary to gain a deeper insight into the pathology of epilepsy require the use of invasive methods or the administration of dangerous chemicals. Since it is not acceptable to subject humans to these trials, it is considered necessary to conduct animal experiments.

Mice are relatively easy to manipulate genetically. By knocking in or out genes, one can make the mouse mimic the pathological state of a disease. Therefore, there are numerous models available that can be applied to research in a wide range of diseases. As mentioned earlier, for research in optogenetics, mice can be genetically modified to express opsins in all or a specific subset of neurons. These genes are often coupled to a reporter gene, i.e. GFP, to make it possible to track where the opsins are expressed. The use of transgenic animals along with the possibility to cross different strains supplies the scientists with an extensive optogenetic toolbox.

All animal experiments in Sweden are regulated by Jordbruksverket and have to be approved by an ethical committee before they are performed.

### **3.4.1 Epilepsy Models**

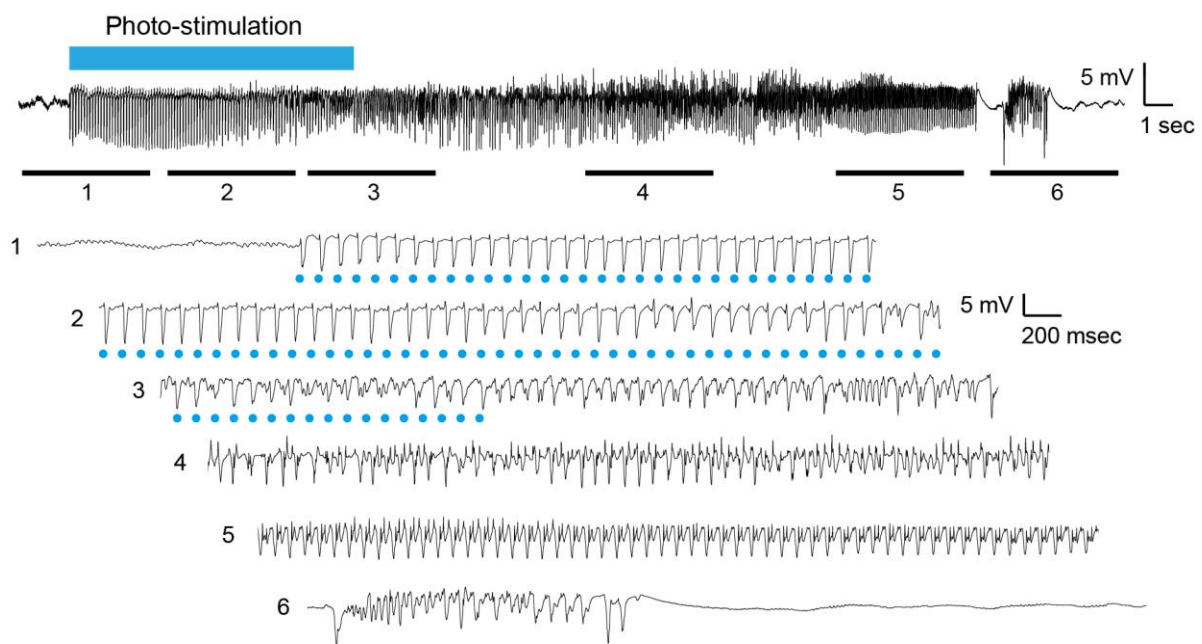
There are multiple animal models used in epilepsy research. They all strive to mimic some feature of the human condition and are used both for basic research of the pathology and in the development of pharmacological agents to treat the disease. There are basically two types of epilepsy animal models. In the first, previously healthy animals can be made to acquire epilepsy by some treatment. Such treatments could be based on chemical compounds or electrical stimulation. The second type of models are genetically predisposed to develop the disease [2].

A frequently used chemically induced model utilizes the excitotoxic kainic acid (KA) to produce seizures of different severity and a seizure focus. The compound, which has a structural similarity to glutamate and interact with the kainate receptor, can be injected by a thin glass capillary into a specific brain region, i.e. the hippocampus. After a couple of minutes, it will produce status epilepticus (SE), a state of continuous seizures. This initial brain insult will, after a latency period, develop into both behavioural and electrographic seizures that arise spontaneously and resemble those of TLE. In addition to the seizures, lesions and sclerosis are also induced by the injection [2, 51].

Another way to develop epilepsy in animals is to use electrical stimulation in a process called kindling. Electrodes stimulate the tissue electrically, preferably in the hippocampus or amygdala. Stimulations are short and repeated according to a schedule. The behavioural manifestations

following such stimulations will increase in both magnitude and length as the stimulations progress [52]. Electrographically, a stimulation is followed by afterdischarges, an EEG pattern that share similarities with TLE seizures. One problem with electrical kindling is the inability to record the neural signals during a stimulation since the provoked currents override the endogenous signals [2].

An optogenetic seizure model would solve the problem of acquiring recordings from the tissue during and immediately following stimulation, but would also provide the option of targeting specific cell types in a much more restricted volume than the electrical kindling does. Current research show that an optogenetically induced spike train is followed by similar afterdischarges as with electrical stimulation (see figure 6). However, no increase in afterdischarge duration or seizure severity (no kindling effect) has been reported [4].



*Figure 6: Optogenetically induced afterdischarges. The neural signalling is initially synchronised with the light stimulation. When the stimulation train comes to an end, the tissue continues to be activated in a fashion similar to an epileptic seizure. Adopted from (Osawa, 2013) [4] in accordance with PLOSone copyright.*

### 3.5 Drugs

The experiments in this project are performed with the animals under anaesthesia for mainly two reasons; to enable insertion of the optrode into the hippocampus and to lessen the suffering of the animal. In this master's thesis two types of anaesthesia are compared to see if there are any effects on the optogenetic seizure model. The compounds involved with the anaesthesia are presented below.

#### 3.5.1 Ketamine / Xylazine

Ketamine is a drug used for anaesthetic and analgesic purposes in both humans and animals, although administration in humans usually is restricted to cases of emergency where breathing depression must be avoided. Though, there are cases where ketamine also has proven to terminate SE [53]. The drug acts on the glutamatergic NMDA receptor as a non-competitive antagonist which



produces a state of dissociative anaesthesia where the patient is relieved of pain but appear to be awake [14, 54]. The potency of ketamine varies between species. Mice need larger doses to achieve satisfactory anaesthesia, at which levels severe breathing deprivation may be fatal. Therefore ketamine is often combined with another sedative such as Xylazine in order to achieve therapeutic effects at lower doses [55].

Xylazine is not administered to humans, but is used for veterinarian purposes only. It is an  $\alpha$ 2-adrenergic receptor agonist which causes sympatholytic effects, such as lowering of blood pressure and sedation, due to decreased sympathetic outflow from CNS to the PNS [14]. Xylazine is suitable for combining with ketamine in order to achieve surgical anaesthesia [56]. An i.p. administration of 80 mg/kg ketamine and 15 mg/kg xylazine is expected to result in 15-30 minutes of surgical grade anaesthesia and will cause sedation for 1 – 2 h [57].

### **3.5.2 Isoflurane**

Isoflurane is a general inhaled anaesthesia that is frequently used in veterinarian surgery. It is used to keep the animal unconscious and is often combined with a local analgesic compound. One considerable side effect is the breathing depression it causes at high doses.

The isoflurane mechanism of action is still debated. The lipid soluble nature of the drug suggests that the compound is incorporated into the lipid bilayers of the cell where it may interact and change the properties of multiple receptors [14]. In general it is hypothesized that it enhances the function of inhibitory receptors such as GABA and glycine receptors and suppress the exciting glutamate, nicotinic acetylcholine and serotonin receptors [58]. In addition to inducing anaesthesia isoflurane is used for treating refractory SE [59]. Isoflurane is fast-acting compared to injected anaesthetics; recovery time in mice is reported to be  $6.9 \pm 3.6$  minutes [60].

## 4. Methodology

### 4.1 The animals

The experiments were performed acutely in transgenic male mice. These were 4-6 month old and bred in the Lund Biomedical Centre (BMC) facility to heterozygotically express Thy1-ChR2-GFP. The founder lines were originally bought from The Jackson Laboratory. All animals had access to food and water ad libitum. This project carried the permit number M206-12 and followed guidelines according to Swedish Animal Welfare Agency regulations.

Two groups of animals were examined; one group was anesthetised with isoflurane only and one group were given a ketamine/xylazine combination just before engaging the stimulation trains. In addition to the two main groups, one animal had sham stimulations under isoflurane anaesthesia (see table 2).

*Table 2. The anaesthetic drug, stimulation light wavelength and number of animals in the experimental groups. The sham stimulated animal is included for comparison.*

<b>Group</b>	<b>Anaesthesia</b>	<b>Light wavelength</b>	<b>Nr. of animals</b>
Isoflurane	Isoflurane	463 nm (in range)	5
Ketamine	Ketamine/Xylazine	463 nm (in range)	4
<i>Sham animal</i>	<i>Isoflurane</i>	<i>593 nm (out of range)</i>	<i>1</i>

### 4.2 Optogenetic stimulation of hippocampus

Optrodes, consisting of an optical fibre and an electrode were inserted in the hippocampus of the mice brain using stereotaxic surgery. A blue 463 nm LED light was guided through the optical fibre to activate the ChR2 expressed in the tissue. The electrophysiological response was measured via the electrode.

#### 4.2.1 Construction of optrode

The optrode was constructed out of a 200  $\mu\text{m}$  in diameter thick optic fibre and a coated 1 M $\Omega$  tungsten electrode. The optic fibre was used as a fundament for the construct in order to stabilize the optrode. Onto the optic fibre, the electrode was super-glued using a compound based on cyanoacrylate. The glue was applied evenly along the optrode, covering the entire surface leaving only the outermost tip of the electrode and fibre. In addition to fixing the electrode to the fibre, two purposes were served by the glue coat. It helped to smooth the surface of the optrode, with the aim of decreasing the trauma to the tissue upon insertion. Also, since the glue is an insulator, it improves the insulation and protects the electrode coating from damages (see figure 7).

The sharp tip of the electrode was placed 0.5 mm below the flat end of the optical fibre. This arrangement is necessary to ensure that the evaluated tissue is adequately illuminated and will respond to the optical stimulation. Only the outermost tip of the electrode is in electrical contact with the environment, which increases the resistance and narrows the measured volume enabling enhanced spatial resolution of the measurement. The high resistance makes it possible to measure the activity from single neurons.

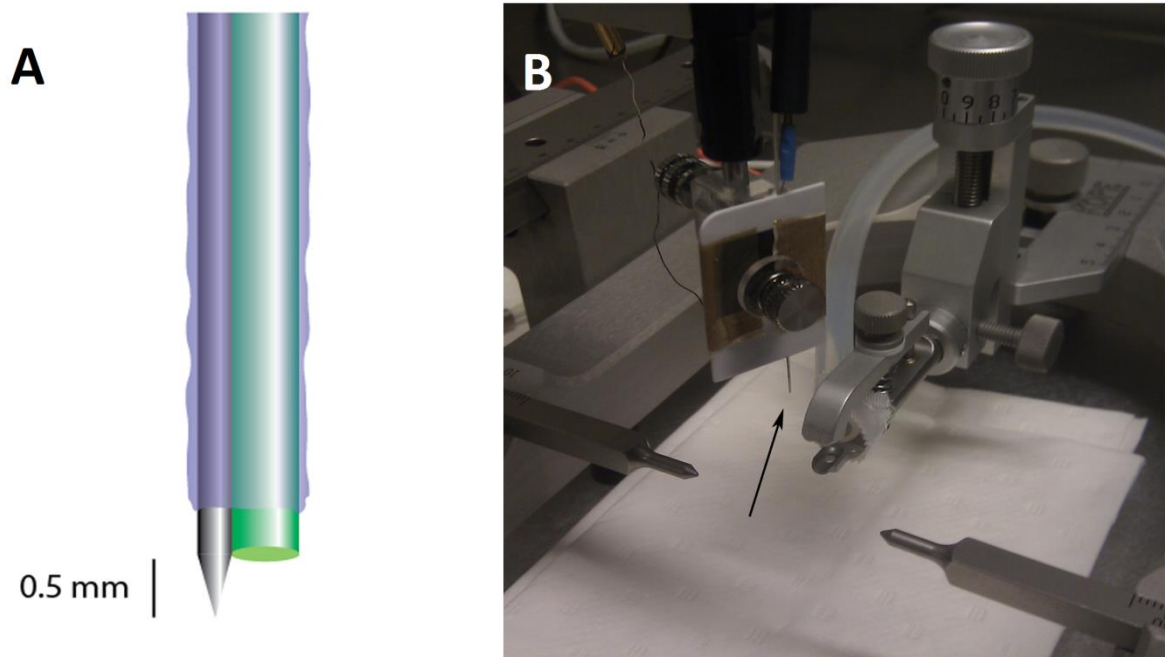


Figure 7. (A) Schematic picture of the optrode tip. The electrode and optic fibre is covered by layer consisting of cyanoacrylate glue. (B) The stereotaxic frame with ear bars and nose clamp. The optrodes position in the stereotaxic frame is indicated with an arrow.

#### 4.2.2 Insertion of the optrode

The optrode was inserted in the hippocampus with the help of a stereotaxic frame to ensure robust localization. The mouse skull was firmly attached to the base of the frame using ear-bars and a nose clamp. Calibration points for the coordinates of the medial to lateral (ML, x-axes) and the rostral to caudal (RC, y-axes) originated from *bregma*. The dorsal to ventral (DV, z-axes) was measured from the *dura*. The coordinates were AP: -3.5, ML: -3.5 and DV ranging from -3.0 to -3.4 depending on the amplitude of the tissue response. These coordinate correlates to the CA1 region of the hippocampus.

After localizing the ML and RC coordinates for penetration, a hole in the skull bone was made using an electric drill. To prevent the *dura* from being dragged along into the brain tissue, it was carefully removed with a needle when the cortex was visible. The ground electrode was placed below the skin in the neck of the animal.

A hydraulic micromanipulator was utilized to control the submersion of the optrode in steps of 0.1 mm every other minute until the desired depth was reached. The response from the tissue was evaluated by each step by the use of two test pulses of light (duration 0.3s, 0.2 Hz).

#### 4.2.3 Measurement setup

The experimental setup was based on the PowerLab hardware (PowerLab 4/35, AD Instruments) which served as a potentiometer with high resolution. The signal from the tissue was first passed through a preamplifier (DP-311 Differential Amplifier, Warner Instruments) which increased the signal amplitude 10 times. Next, the PowerLab hardware digitalized the signal and relayed it via an USB-cable to the PowerLab software in a laptop computer where it was recorded.

The PowerLab and the LabChart software were also used to control the light stimulation. The output from the PowerLab was triggered by a collection of stimulation macros (automatic protocols) in the software. One channel was reserved to record these output signals in time correlation to the tissue response. The output from the PowerLab, in turn, was connected to the power source controlling the stimulation LED. This setup enabled setting the duration and frequency of the pulses by macro control from the software. Light intensity was altered by changing the output power on the LED power source.

The stereotaxic frame and all incoming cables were housed in a 1 m<sup>3</sup> grounded faradays cage to minimize the noise (see figure 8).

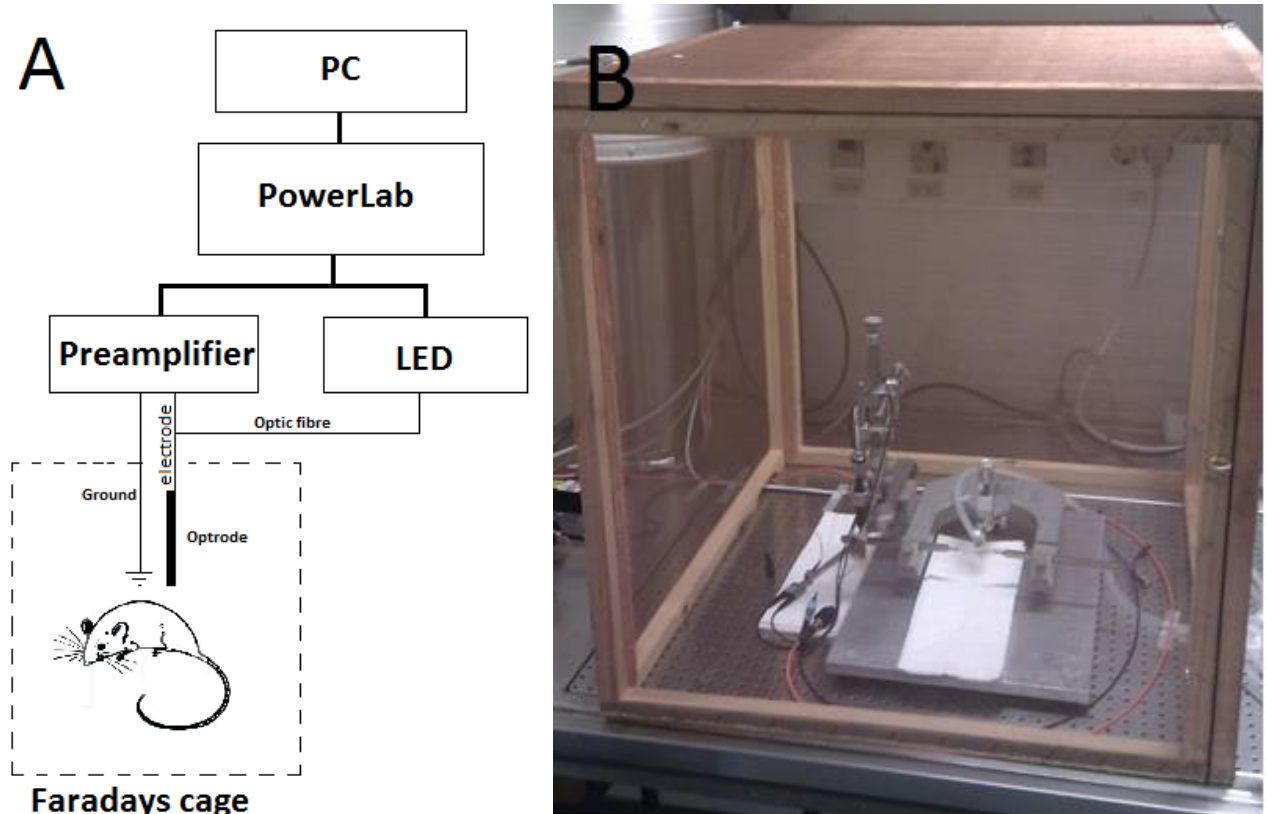


Figure 8. (A) Schematic illustration of the experimental setup. The PowerLab control both the recording and the light stimulation. (B) The experimental setup was enclosed in a faradays cage to minimize noise.

#### 4.2.4 Stimulation protocol

The light stimulation of the tissue was controlled by macros in the LabChart software. These macros independently engaged stimulation trains at predetermined time intervals. A train had a 20 second duration and contained 5 ms light pulses fired at a rate of 10 Hz. These trains were activated once every 5 minutes. The amount of trains was 30 at a minimum, but the number was increased if the animal showed potential of developing more afterdischarges. The entire procedure lasted typically for 4 h. including the pre stimulation surgery.

Stimulations were performed by a blue LED light (UHP-LED-460, Prizmatix) emitting a wavelength of 463 nm which is within the activation range of the ChR2. The output effect from the optrode tip was measured with an optical power meter (Newport, Optical Power Meter 1916-C) and calibrated to

hold 2.3 mW which would yield a power of  $75 \text{ mW} / (\mu\text{m})^2$ . Too high output power would damage the tissue because of heating [61].

Also, one animal was sham stimulated with laser (CrystalLaser, GLC-025-593) holding a wavelength of 593 nm, which is outside the ChR2 activation range (see figure 9).

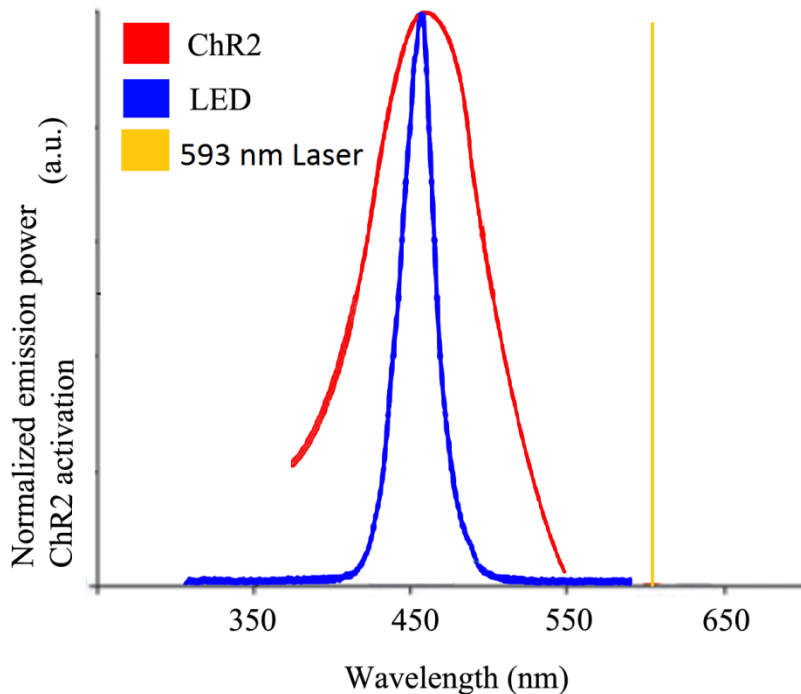


Figure 9: The LED emission spectra and ChR2 activation spectra overlaps sufficiently. The 593 laser, on the other hand, will not activate the ChR2.

#### 4.2.5 Anaesthesia

The primary anaesthesia was isoflurane administered by an automatic pump (410 Anaesthesia Unit, Univentor). For induction, a chamber was filled with a 5% partial pressure of isoflurane. When the animal was asleep it was transferred to the stereotaxic frame where a tube connected to the anaesthetic pump was located in the close proximity of the nose. Anaesthesia was maintained with a dose of 0.7 -2.0 % isoflurane partial pressure and was frequently adjusted to the appropriate level. Paw withdrawal response and breathing patterns were checked to approximate the anaesthetic level of the animal.

For the isoflurane group, isoflurane anaesthesia was maintained during the entire experiment. In the ketamine group, the isoflurane anaesthesia was exchanged to ketamine/xylazine after the initial surgery and optrode implantation was finished. When switching to the ketamine/xylazine anaesthesia, a combined dose of 80 mg/kg ketamine and 15 mg/kg xylazine was administered i.p. just before the isoflurane pump was turned off. A booster dose of only ketamine (80 mg/kg) was administered after approximately 1.5 h.

In order to support the animal body temperature, the experiment was performed with the animal lying on a heating pad. The pad lost temperature after about 1.5 h and had to be replaced with a new one.

### 4.3 Histological analysis

Immediately following the experiment, after a large euthanasic i.p. injection of sodium pentobarbital, the mice were perfused with 0.9% saline followed by 4 % paraformaldehyde (PFA). The brain was retrieved and put in PFA for 24h. Next, the brain was transferred to a solution of 20 % sucrose for another 24 h incubation. 30  $\mu\text{m}$  slices were cut by freezing the brain with dry ice and placing it in a microtome. Four series were obtained each containing about 30 slices. One of the series were mounted onto hydrophobic microscopy glass and fixed by DABCO solution with added H $\ddot{o}$ chst staining before covering with a cover glass. The other samples were stored at -20°C.

The first brains were cut using coronal slices but later horizontal slices were used because of the higher probability of covering the entire lesion.

The slices were examined in a microscope (BX61, Olympus), looking at the lesion created by the optrode. Blue light was used to excite the GFP in order to look at the Chr2-GFP expression and UV light was used to visualise the H $\ddot{o}$ chst stained nuclei. The actual location of the optrode tip was determined by comparison to the mouse brain atlas [62].

### 4.4 Data evaluation

The analysis of the recorded data was performed in LabChart, MatLab and MS Excel.

Values are presented as: mean (1 s.d.) throughout the paper and differences considered significant if  $p < 0.05$ .

#### 4.4.1 Afterdischarge detection

Because of the variability and fluctuations of the data, a method to track the development of afterdischarges was designed. The method utilizes filtering functions and mathematical algorithms in the LabChart software. First, the recording from the hippocampus was band-pass filtered between 2 – 250 Hz and notch filtered for 50 Hz. Subsequently the root mean square (RMS) value of each 10 second period immediately following the stimulation trains was calculated. This value was then compared to an artifact free, 10 second long, period preceding each stimulation by calculating an activity ratio.

$$\text{Activity ratio} = \frac{\text{RMS after stim.}}{\text{RMS before stim.}}$$

The activity ratio will be larger than 1 if the post stimulation activity exceeds the pre stimulation activity. In case of an afterdischarge, the value will be much larger than 1.

The RMS is calculated by:

$$\text{RMS} = \sqrt{\frac{1}{n}(x_1^2 + x_2^2 + \dots + x_n^2)}$$

where  $n$  is the number of data points and  $x$  are the values. The equation will return an estimate of the deviation magnitude of the data in relation to a zero baseline. Because of the band-pass filtering, the EEG is oscillating around 0.

#### 4.4.2 Burst suppression rate

A common EEG pattern that emerges when measuring the brain activity in an anaesthetised brain is the burst suppression rate (BSR). The pattern is comprised of bursts of activity separated with an isoelectric EEG (flat line). This pattern is observed in heavily sedated patients and correlates to some extent to the level of isoflurane anaesthesia, which has been shown in rats [63].

A short algorithm in MatLab was used to detect activity above a threshold of 0.25 mV and to calculate the BSR (see figure 10). If the threshold was exceeded, 350 ms of activity is noted. The duration is prolonged if there are more peaks following the first (see figure 11). The BSR can then be calculated as the percentage of time spent in isoelectric state.

$$BSR = (100) \frac{t_{total} - t_{active}}{t_{total}}$$

where  $t_{total}$  is the duration of the analysed time and  $t_{active}$  is the cumulated time spent in bursting state.

```
last = 0;           %The index of the last detected peak
burst = [];        %Vector with detected activity
eeg;              %The EEG data with 1 ms resolution
t = 0.00025;      %Threshold for burst detection (V)
step = 350;       %Time reserved to count as active (ms)

for i = 1:length(eeg)
    if (eeg(i) < -t || eeg(i) > t);
        burst(i:i+step) = 1;
        last = i;
    else if (i - last) > step
        burst(i) = 0;
    end
end
end
```

Figure 10: MatLab algorithm for detecting the periods with bursts from an EEG recording.

## 5. Results

This section first addresses the direct tissue response from the light stimulations. Secondly, events that occur outside the stimulation periods are presented. Finally, there is a section dealing with the histologic analysis following the experiments.

### 5.1 Test pulses

Stimulating the Thy1ChR2 mice with 463 nm LED light immediately evoked a response from the tissue in both the isoflurane and ketamine group. A 300 ms light pulse resulted in a fast spike at the beginning, followed by a slight decrease in potential to a level that was maintained during the light pulse (see figure 11). The amplitude of the spike varied as the optrode was lowered into the tissue.

The averaged response potential, just before the first train stimulations, was -4,2 (1.0) mV (n= 9) considering the total of the animals. There was no difference between the groups ( $p = 0.06$ , two-tailed unpaired Student's t-test, see table 3). No response could be seen in the sham stimulated (593 nm) animal.

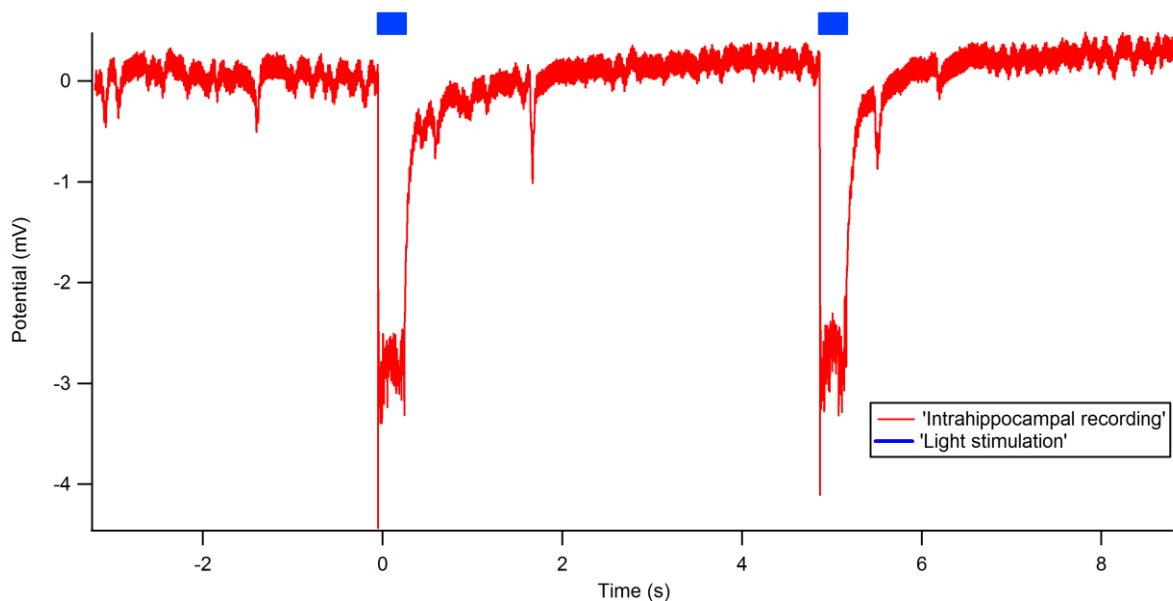


Figure 11: The response to two 300 ms long consecutive light stimulations. The light pulses are indicated by the blue bars and the EEG recording with a red trace. There is a -4.5 mV peak potential upon stimulation.

Table 3: The response from the test pulses.

Group	Response [mean (1 s.d.)] (mV)
Isoflurane n=5	-3.68 (0.86)
Ketamine n=4	-4.9 (0.75)
Total n=9	-4.2 (1.0)



## 5.2 Stimulation train

A 20 second long train of 5 ms pulses in 10 Hz resulted in spikes corresponding to the stimuli frequency. The amplitude of the spikes were initially large, starting at the same level as the 300 ms stimulations, and then decayed and stabilized at a level corresponding to about half of the initial value (see figure 12).

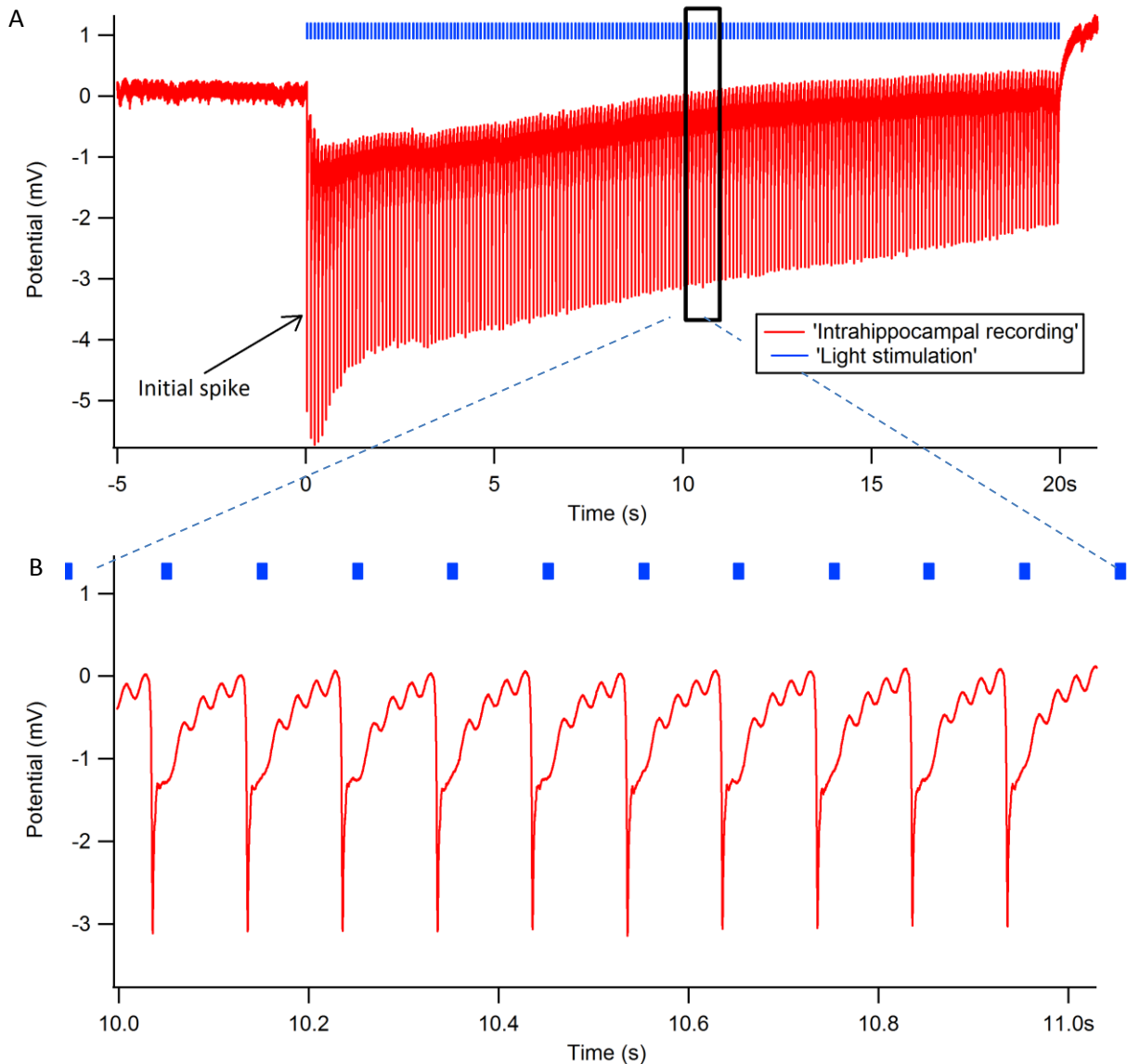


Figure 12. (A) A intra hippocampal recording during a spike train stimulation with the initial spike indicated by an arrow. The tissue responded in phase with the stimulations but decreased in amplitude at the end of the train. (B) An enlargement of the area in the black rectangle in figure 13A. A small amplitude 50 Hz oscillation superimposed on the spikes is visible.

The amplitude of the initial spike in the stimulation trains changed during the experiment (see figure 13). The voltage values rise and fall in a continuous manner even though the general trend seems to be that the amplitude decrease with the number of stimulations.

The decrease was estimated by averaging the amplitude of the initial spikes from two periods of the experiment; stimulation number 1-5 and number 25-29. The values from these calculations were

then averaged for the two trial groups (see figure 14). There is a significant decrease when looking at the total of the animals (paired Student's t-test,  $n=8$ ) even though there is no significance within or between the trial groups (unpaired Students t-test,  $n = 5$  isoflurane,  $n = 3$  ketamine). The decrease in amplitude was 0.78 (0.73) mV when considering all of the animals. One animal (#K4) was excluded from the ketamine group due to corrupt data.

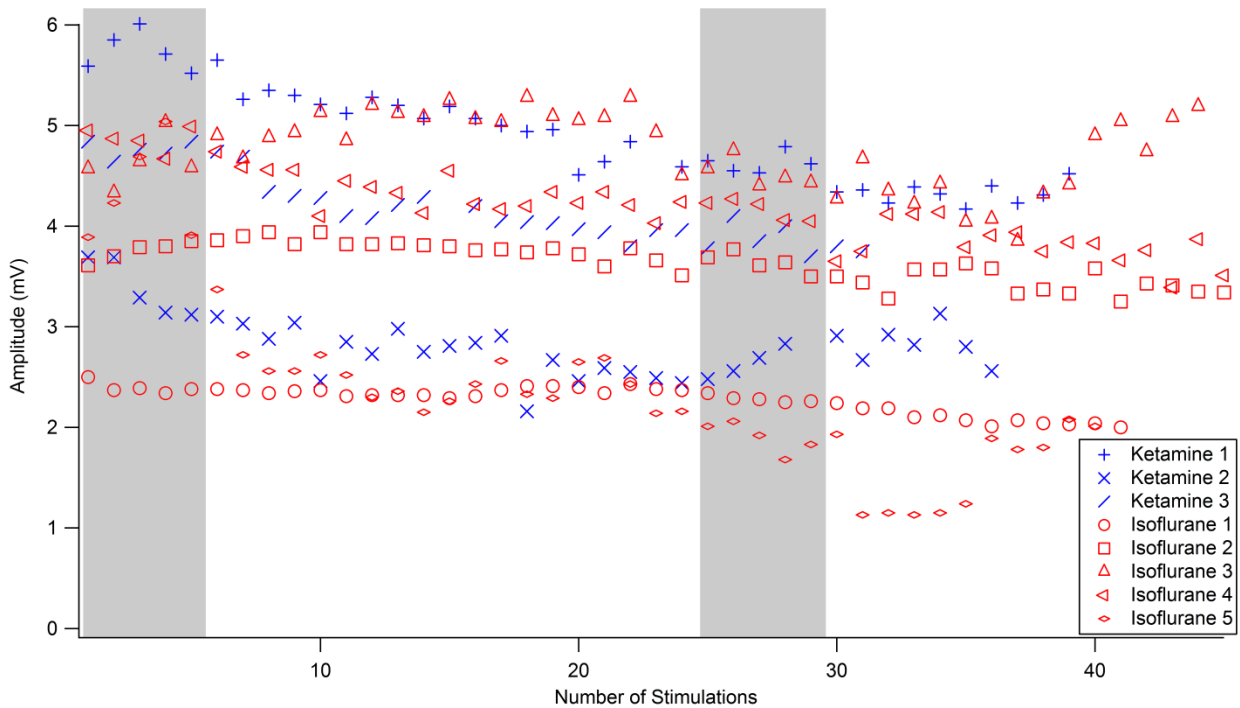


Figure 13. The amplitude of the initial spike in a stimulation train. The level of the amplitude differs between animals and there is a general decrease in amplitude following multiple stimulations. The compared periods (stim. nr. 1-5 and 25-29) are marked in grey.

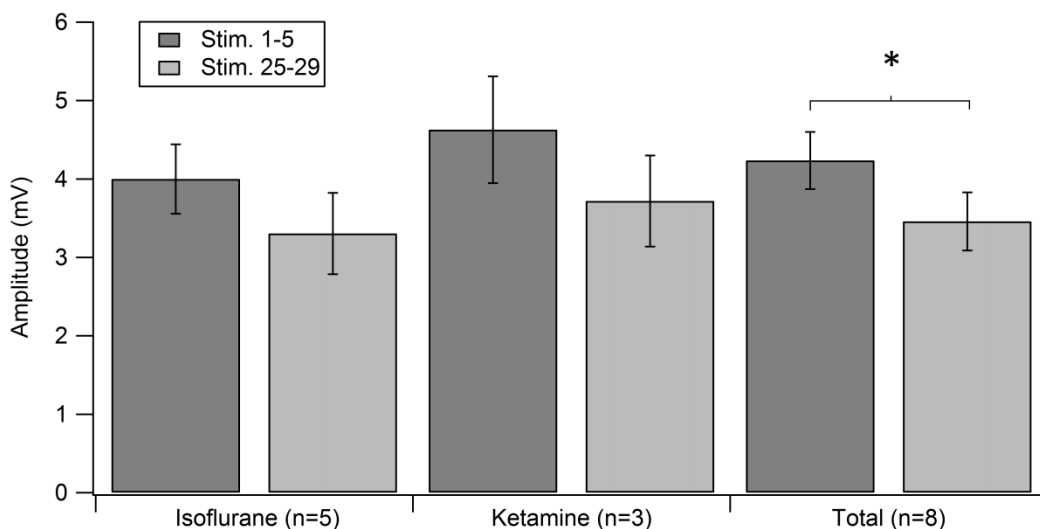


Figure 14. The averaged amplitude of the initial spikes from 5 stimulations in the isoflurane group, the ketamine group and the total of the animals. Two periods are compared; stim. nr. 1-5 and nr. 25-29. There is a significant difference between the two periods when considering all of the animals. The error bars indicate 1 standard error of the mean (SEM).

### 5.3 Afterdischarges

At some occasions, especially in the second half of the experiment, the stimulations could be followed by self-sustained repetitive discharges. They tended to first appear alone and then to increase in number, in some animals peaking at a total number of ten. Although the appearance and form of the afterdischarges differed between the animals they all appeared within 15 seconds following a stimulation train (see figure 15).

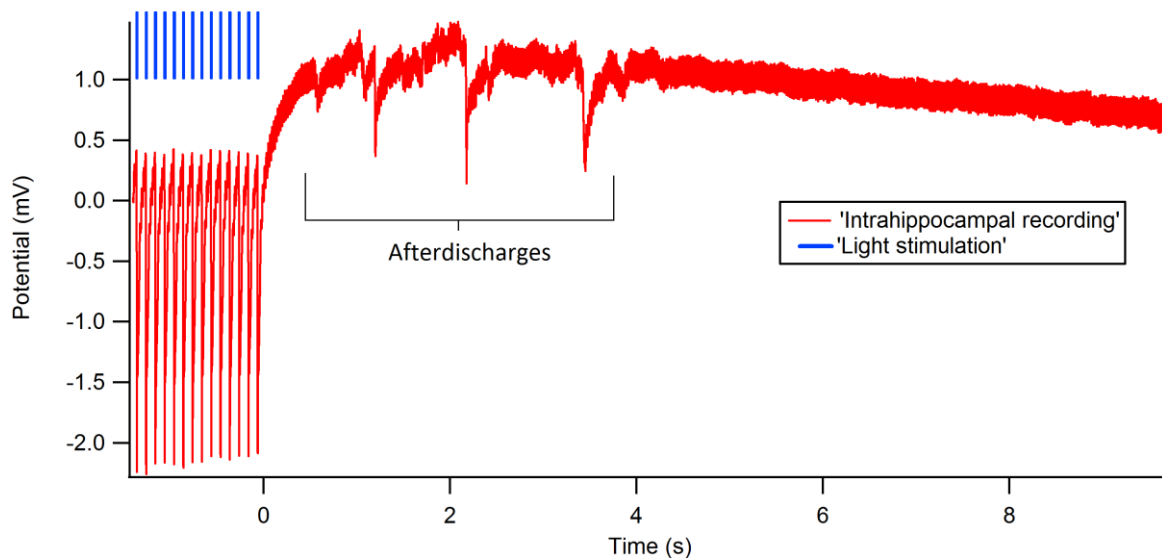


Figure 15: Typical afterdischarges following a stimulation train in an isoflurane anesthetized animal. Light pulses are indicated with blue bars.

The evaluation method based on the RMS values was used to measure the afterdischarge activity ratio. In most animals the activity ratio was lower than 1 following the first trains, indicating a depression of neural activity after stimulation. In the isoflurane anesthetised animals, this period of suppressed neural activity post stimulation, seemed to cease after about 25 stimulations and instead afterdischarges tended to appear in varying amount. The ketamine animals, on the other hand, did not develop afterdischarges.

The values were fluctuating within the single animal even though there seemed to be an underlying continuous mechanism that gradually increased the amount of afterdischarges. In figure 16, one isoflurane, one ketamine and the sham stimulated animal are presented. The isoflurane animal showed low activity in the beginning and then gradually began to develop afterdischarges around stimulation 20. In the ketamine animal, no afterdischarges could be detected, even though there are two values indicating an elevated activity. The sham stimulated animal was fluctuating around the value of 1.

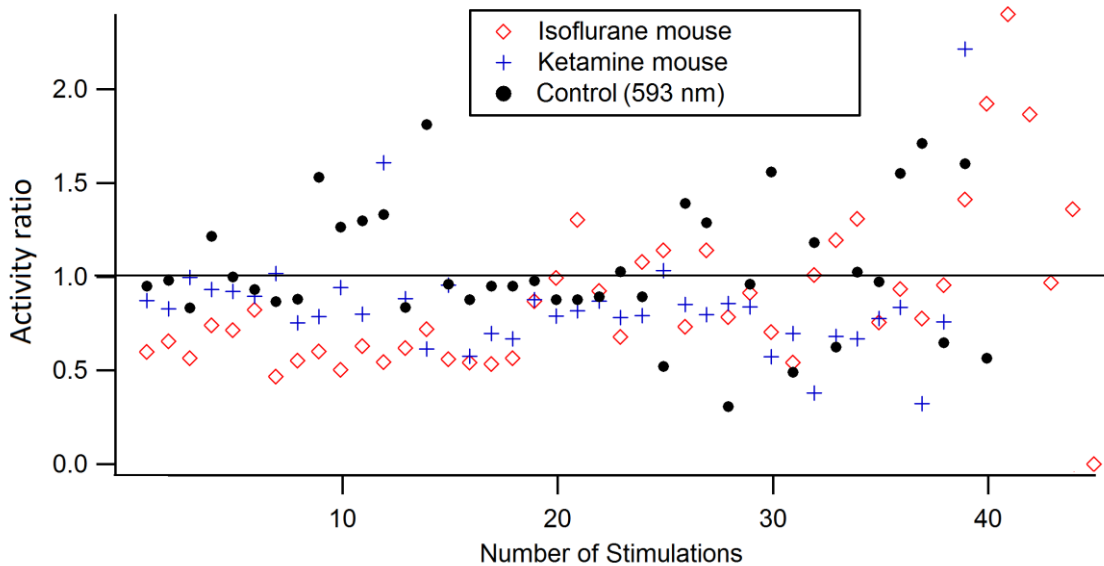


Figure 16: Afterdischarge activity ratio in three animals. The isoflurane animal (#5) develops afterdischarges after stimulation nr. 20. The ketamine animal (#1) does not have any afterdischarges. The animal with the off-range wavelength sham stimulations varies around 1.

As indicated by figure 16, the start values of the individuals differed. The isoflurane animal showed more activity depression following stimulation than the ketamine and sham stimulated animal during the 5 first stimulations. Since the start level varies in between animals it is also interesting to evaluate the relative development of the activity ratio in each animal. The relative development was calculated by dividing all the values with an average of the first 5 stimulations. This procedure normalised the groups and made it possible to compare them by average and standard deviation. The result is presented in figure 17.

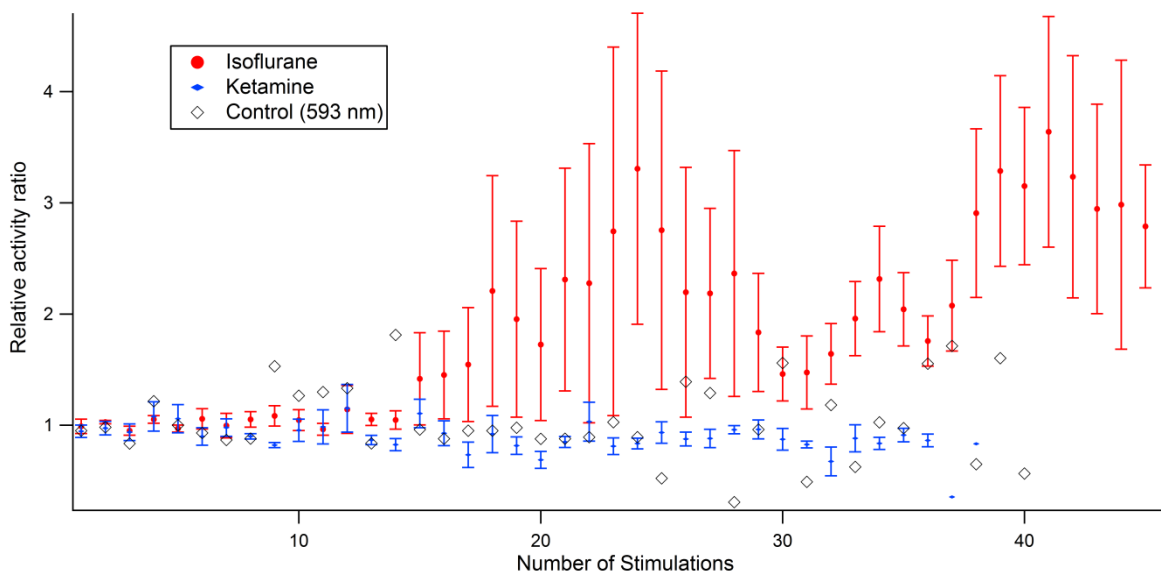


Figure 17: The averaged relative activity ratio in the isoflurane and ketamine group. (isoflurane  $n = 5$ , ketamine  $n = 4$ ). The isoflurane animals show a rise in activity with a large divergence in the data. There is no indication of an increased afterdischarge activity in the ketamine animals. The sham stimulated animal is included for comparison. Error bars indicate 1 SEM.

Reviewing figure 17, it is clear, that the ketamine animals do not show an increase in activity ratio following the stimulations, meaning that no afterdischarges did emerge. The isoflurane anaesthetised animals, on the other hand, begin to develop afterdischarges around the 20<sup>th</sup> stimulation but the values are variable. The sham stimulated animal fluctuates around 1.

Because of the low number of animals used in this study, it is hard to show any significant difference considering only one stimulation number. Though, if the values are gathered into bins of 5 stimulation events, a significant difference between the isoflurane and ketamine group can be showed in bin 16-20, 21-25, 26-30, 31-35 and 36-40 (unpaired Student's t-test, see table 4). In addition to the difference between the trial groups, there is a significant increase between the isoflurane bin 16-20 and 36-40 (see figure 18) (paired Student's t-test, see table 4).

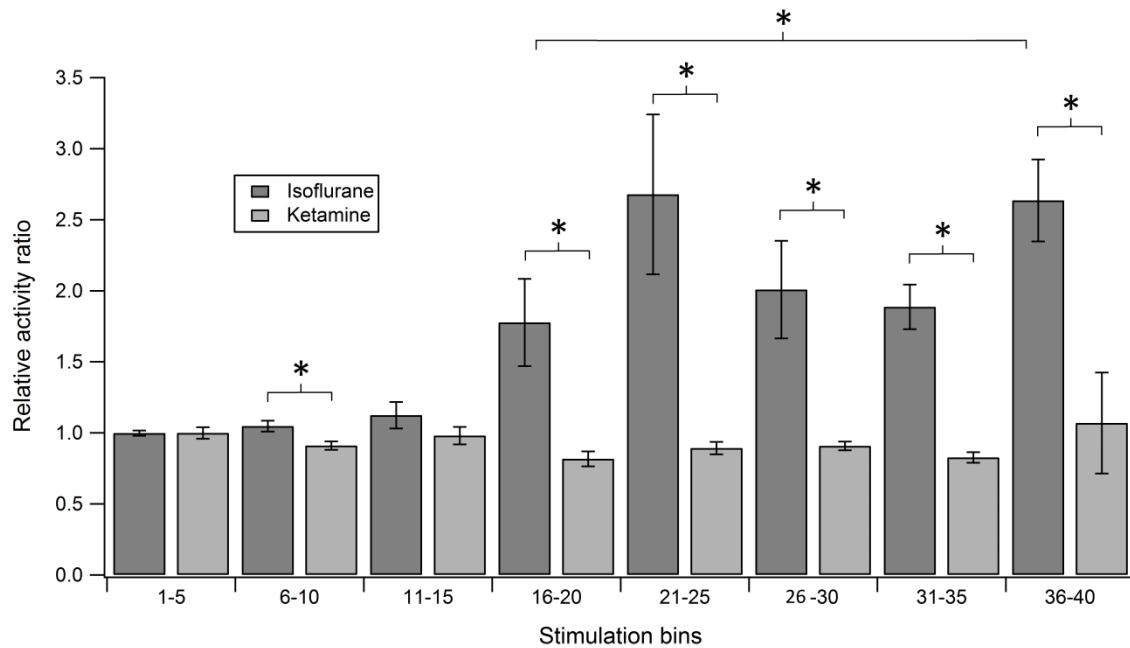


Figure 18: The relative activity ratio gathered into bins of 5 stimulations. The isoflurane group show an increase in activity following stimulation 16, and the ketamine group remains at a level around 1. There is a significant difference between the two trial groups, and also within the isoflurane group, as indicated by asterisks.

Table 4: The number (n) of measurement points used for the Student's t-test in figure 18.

Bin	1-5	6-10	11-15	16-20	21-25	26-30	31-35	36-40
Isoflurane	25	25	25	25	25	25	25	25
Ketamine	20	20	19	19	20	19	16	5

As there seem to be some correlation between the number of stimulations and the occurrence of afterdischarges in the isoflurane group, a Pearson's test was performed (see table 5). The test show significant linear correlation in 4 out of 5 animals.

Table 5: Result from the Pearson's correlation test comparing the number of stimulations with the activity ratio. The result indicates a linear correlation in 4 out of 5 isoflurane animals.

Animal	R	R2	p
Isoflurane 1	0,657	0,432	*
Isoflurane 2	0,831	0,691	*
Isoflurane 3	0,601	0,361	*
Isoflurane 4	0,143	0,021	NS
Isoflurane 5	0,632	0,400	*

## 5.4 Spontaneous events

In addition to afterdischarges there were larger events on the recording that appeared spontaneously without any common rhythm (see figure 19). These events occurred in varying amount in all animals and differed widely in duration. The abundance of these events rose and fell throughout the experiment and they were in some animals dominating the recording. Spontaneous discharges were seldom detected immediately after a stimulation train.

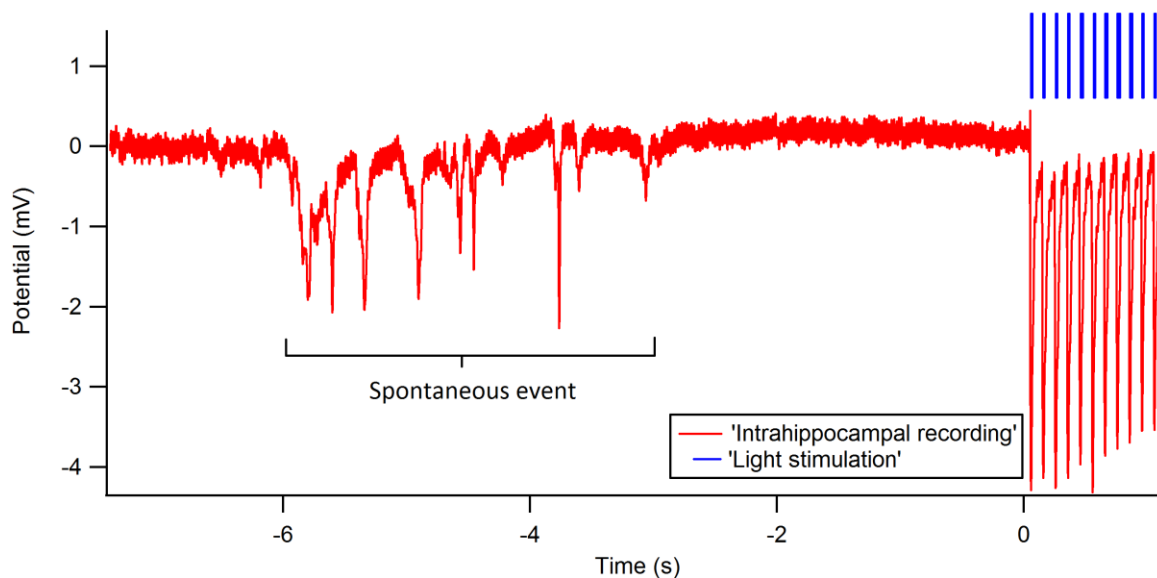


Figure 19. A typical spontaneous event, in this case occurring just before a stimulation train.

The amount of spontaneous activity can be appreciated by calculating the BSR. Because of ubiquitous breathing artifacts in the ketamine group no BSR for these could be calculated. Below, in figure 20, 3 animals representing standard cases are presented. The 'Isoflurane animal 1' has a BSR above 95% during the entire procedure. Both the 'Isoflurane animal 2' and the sham stimulated animal have a period with more spontaneous activity in the middle of the experiment.

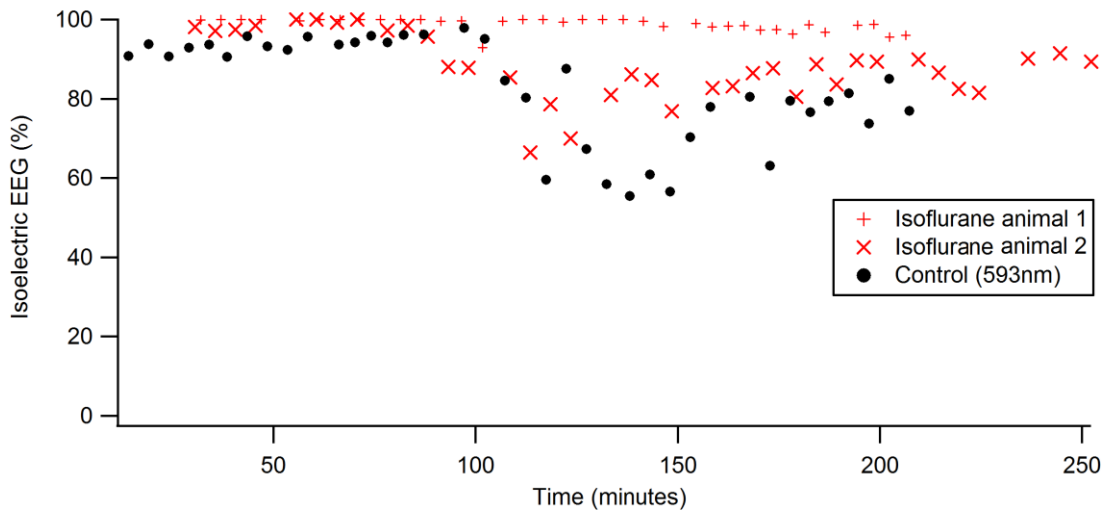


Figure 20. The BSR (presented as the percentage of time spent in isoelectric EEG) of two isoflurane anesthetized animals and the sham stimulated animal. The time is counted from the start of the experiment. Both the 'Isoflurane animal 1' (#14) and the sham stimulated animal show an increase in spontaneous activity around 120 minutes after the start of the experiment. In contrast, the 'Isoflurane animal 2' (#11) is inactive during the entire procedure.

In order to see if there is a correlation between the BSR and the occurrence of afterdischarges, a Pearson's correlation test was performed within the isoflurane group. The results show correlation in 2 out of 5 animals (see table 6).

Table 6: Result from the Pearson's correlation test comparing the BSR with the activity ratio. The result indicates a linear correlation in 2 out of 5 isoflurane animals.

Animal	R	R2	p
Isoflurane 1	-0,428	0,183	*
Isoflurane 2	-0,466	0,217	*
Isoflurane 3	-0,162	0,026	NS
Isoflurane 4	-0,084	0,007	NS
Isoflurane 5	-0,322	0,104	NS

## 5.5 Optrode localization

In order to gain a high response to the light stimulations it was essential to place the optrode at a position where there was a high expression of ChR2. There are quite large structural damages along the path of the optrode but at the outermost tip of the electrode the tissue is almost intact. All optrodes were located in the CA1 region or at the border to the dentate gyrus. Figure 21 and 22 below show the optrode position in two animals. Intense green correlates to expression of ChR2-GFP.

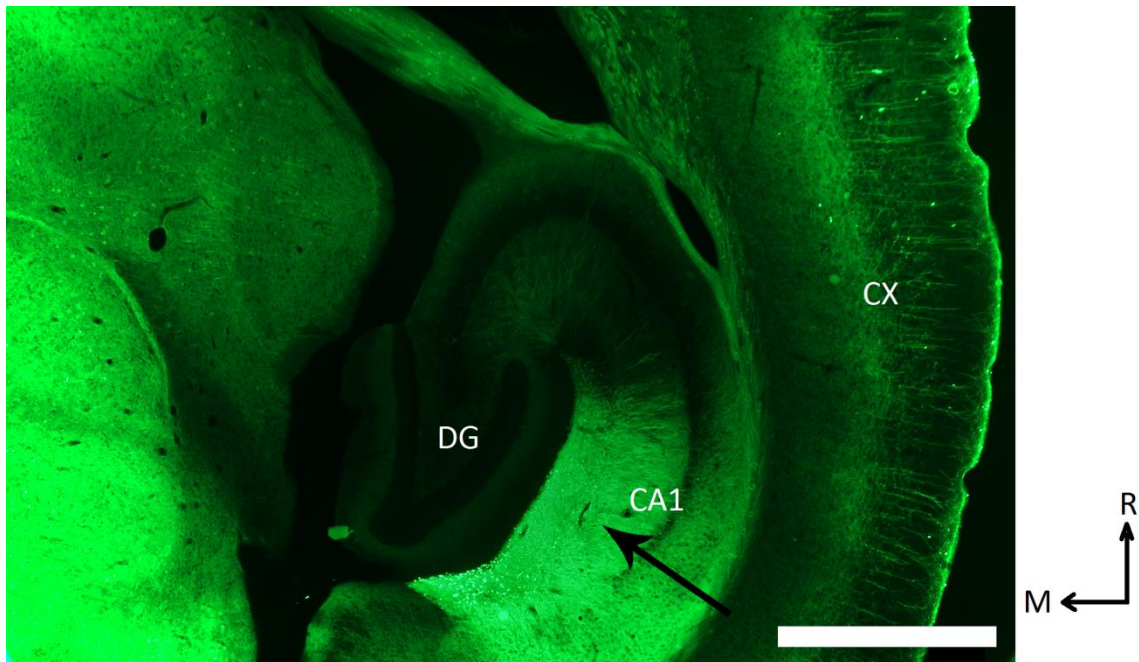


Figure 21. Horizontal section of the hippocampus. The arrow indicates where the optrode was located. Scale bar: 800  $\mu\text{m}$ .

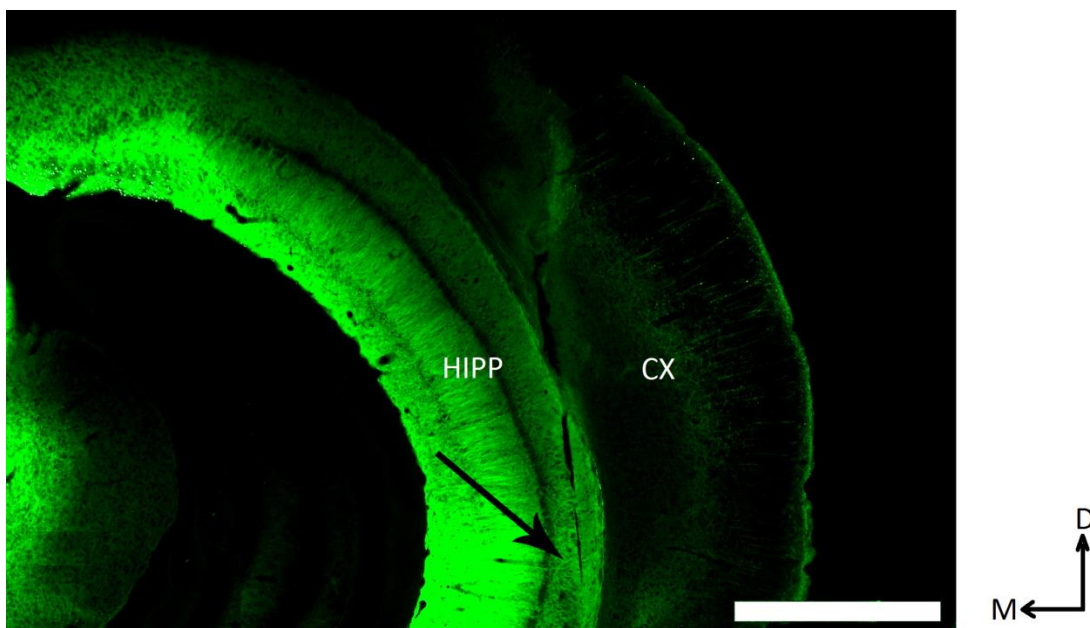


Figure 22. Coronal section of hippocampus. The arrow shows where the tip of the optrode was located. Scale bar: 800  $\mu\text{m}$ .



## 6. Discussion

In this section, the results from the experiments are discussed in the order they are addressed in the results section. Thoughts about how optogenetics can be applied in epilepsy and in other novel ways are also presented. As it is not uncontroversial with gene therapy and animal experiments, a section with an ethical discussion is included.

### 6.1 Evaluating the results

What is prominent in this project is the individuality in the recordings from the animals. Each animal has its own sequence of events and its own characteristics. This is of course expected considering the diversity of how individuals respond to drugs [14] and the plasticity of the brain [16].

What adds to the difficulty in analysing the data is the abundance of artifacts throughout some parts of the recordings. These mainly come in two types; breathing artifacts and electronic artifacts. The former are related to the in- and exhalation of the animal and is of course dependent on the breathing rate and depth. Because of the depressing action isoflurane has on the breathing drive, the artifacts coupled to the ventilation in the isoflurane animals is limited, but the ketamine anaesthetised animals are heavily affected.

The breathing artifacts are characterised by periodic broad spectrum peaks in the EEG recording. Because of its broad-range frequency components it is difficult to filter them out without losing other valuable peaks. It is hard to tell how the artifacts found their way into the recording. One explanation could be that it is a slight movement of the ground electrode position or some muscle related electric impulse leaking into the electrode. Ketamine anaesthesia, at the dose used, proved unsuitable for the optogenetic seizure model since it is crucial to be able to distinguish low amplitude peaks with high time resolution.

The electric artifacts came from large amplitude electrical oscillations interfering with the measurements. At some occasions the problem was located to improper grounding, but sometimes this large noise seemed to be idiopathic. Luckily, these periods of disturbance are restricted in time and were disregarded when analysing the data.

As seen in figure 13A, some 50 Hz power net oscillations were sometimes visible in the EEG. This noise had low amplitude and could be filtered out by the LabChart software, hence not affecting the quality of the EEG.

#### 6.1.1 Test pulses

Overall, the results of the optogenetic stimulations were robust and good. The Thy1-ChR2 gene delivered large depolarisations in response to the short stimulations, with a magnitude of nearly 10 times the background neural signalling. This indicates that a large population of neurons fired simultaneously in response to the blue light in the volume surrounding the optrode tip. There were no stimulation artifacts, as there are when using electrical stimulation, which was confirmed by the isoelectrical signal obtained during the 593 nm light trains in the sham stimulated animal (data not shown).

The amplitude of the response varied as the optrode was lowered into the tissue. One likely explanation is that there are local differences in ChR2-GFP expression, which is confirmed by the histological analysis.

A question that emerges is whether the anaesthetic compound directly interacts with the opsin. Since both isoflurane and opsins have their site of action in the cell membrane it is not farfetched

that there could be some kind of interaction. Though, in order for ketamine to interfere with the opsin, some kind of binding and steric hindrance would be required. The result from the short stimulations did not indicate any difference (even if close) between the ketamine and isoflurane group considering tissue response. But more studies, designed for the specific cause of finding drug-opsin interaction, are needed to draw any definitive conclusions. Of course, one challenge with such study would be to stimulate the ultimate control group, conscious animals that are not under drug influence.

### **6.1.2 Stimulation train**

The recordings from the stimulation trains confirm the high speed resolution of the Chr2 [37]. These stimulations clearly made such impact on the neurons that afterdischarges were formed in the isoflurane group.

The amplitude of the stimulation spikes decreased during the experiment, as described in both the ketamine and isoflurane group. Of course, the animal stimulated with 593 nm light cannot contribute to this particular analysis because of the absence of peaks.

One possible explanation to the amplitude decrease could be exhaustion of the neural tissue. Heavy signalling due to the stimulations and/or the time spent under anaesthesia could have impact on the neural performance. It is commonly accepted that small animals need support with maintaining body temperature during surgery. The high surface to volume ratio and low muscle activity result in a temperature drop that might have an impact on CNS metabolism [64].

The amplitude level also differed between the animals as seen in figure 13. This could be due to tissue damage or different expression level in the vicinity of the optrode tip.

### **6.1.3 Afterdischarges**

Much effort has been put into finding the cause of what parameter or parameters that influence the development of afterdischarges. It is clear that ketamine, at the given dose, blocks the afterdischarges effectively. Since ketamine works as an antagonist on the NMDA receptors [54], one can reasonable speculate, that these are involved in the development of afterdischarges. As mentioned earlier, ketamine has been reported to end SE.

The appearance of afterdischarges varied much between the isoflurane animals. In some individuals there were very few afterdischarges while one animal developed massive activity lasting for up to 20 seconds. For example, the peak seen in figure 17 around stimulation nr. 23, is mostly composed of a massive increase in afterdischarges in one single animal. The animal was not excluded from this analysis in order to point out this diversity in afterdischarge occurrence. What caused these fluctuations is not completely clear when reviewing the results. An analysis of linear correlation in each isoflurane animal show that there is a very low correlation between the BSR and the appearance of afterdischarges in the isoflurane anaesthetised animals. On the other hand, there is a trend that the amount of afterdischarges increases with the number of stimulations. This indicate that there might be a kindling effect, in contrast to what is recently reported [4].

### **6.1.4 Spontaneous events**

As mentioned earlier, the BSR could not be calculated for the ketamine group. The breathing artifacts had such magnitude and spectrum that they dominated the recording and overshadowed the relatively low magnitude spontaneous events.

The results from the isoflurane group on the other hand showed that the amount of spontaneous activity gradually increases and decreases in a continuous manner. There is a lot of individuality among the animals; some had almost a complete isoelectric baseline during the procedure while others had periods where there were more bursts than flat-line.

One early hypothesis was that the spontaneous events somehow were induced by the stimulation trains and represented a kind of inter ictal events. Though, if this were true, the sham stimulated animal would have no spontaneous activity, which is not the case (see figure 20). Also, the linear correlation analysis comparing the BSR and the afterdischarge occurrence showed very low dependence between the variables (see table 6).

Another possible explanation, which is supported by the literature, is that the spontaneous burst pattern is related to the level of sedation [63]. A period of more bursting would then indicate a more awake animal. The continuous fluctuations in the BSR could then be related to the adjustments of anaesthesia during the experiment or the changing of the heating pad.

Isoflurane uptake in mice is to some extent self-regulatory since a high dose depresses the breathing rate, and the uptake is dependent on the breathing. In an experimental situation the breathing rate is monitored and used as an indication of whether to adjust the dose. After the adjustment there is latency until the drug has reached an elevated level in the CNS compartment. This could explain the continuous variation in the BSR.

As this project focused on the qualitative aspects of isoflurane versus ketamine anaesthesia there are too few data points from the isoflurane dose adjustments to draw any conclusions from the BSR dose dependence, even though there is an indication that there is a correlation (data not shown). The method for evaluating the BSR could be used in future studies designed to study such dose dependence.

### **6.1.5 Optrode localisation**

In this project the histological analysis confirmed that the optrode tip was located in the CA1 region. Though, it is likely that the response from the tissue is dependent on the particular ChR2-GFP expression in the immediate vicinity of the optrode tip. The amount of expression could be assessed by studying the intensity of GFP emission from the tissue when examining the samples in the microscope. In this case, the preparation of the samples must be exactly the same to be able to compare the emission intensities.

What could be also important is if the optrode tip was introduced immediately over the pyramidal cell layer or over an area with processes. The former alternative would yield a better response because of the higher density of cells.

The trauma caused by the insertion of the optrode could also affect the response from the tissue and have an impact on the individual variations in the animals. Intact undamaged tissue holds more healthy cells that are able of responding to optic stimulation. The amount of damage could, in a future assay, be evaluated by immunostaining.

## **6.2 Optogenetics and epilepsy**

This work has focused on stimulating neurons to provoke epileptiform activity. There are however indications that optogenetics can be used to treat seizures as well [26]. There are multiple subclasses of neurons in the hippocampus. Many of these can be targeted using specific promoters and Cre-LoxP techniques [61]. It is speculated that successful dampening can be achieved either by inhibiting

principal cells by NpHR or exciting interneurons using ChR2 [36]. Of course this is closely related to the basic research of finding the cause(s) of epilepsy.

As inhibitory optogenetics have proven to dampen the activity of neurons [65], it is obvious that the technique is of interest when searching for novel epilepsy treatments. Ideally, a refined clinical solution should have a sensing unit that continuously record EEG data and evaluates it for epileptiform activity. If such activity would take place, the optical treatment is to be activated until the seizure has ended.

When analysing EEG data for such on-demand treatments, one must understand that it is fluctuating and changing nature depending on the situation. In a healthy person, the brain activity varies during different stages of sleep and if the patient is awake different patterns may emerge depending on activity [16]. Some physiological patterns even may resemble that of epileptiform activity. Hence, there is a need for delicate signal processing if a seizure is to be detected within milliseconds. One interesting approach is the use of an artificial neural network (ANN) [66]. These networks are capable of recognizing complex patterns in fast manner. The downside, though, is a long learning phase which requires time and processing power. Another approach is using mathematical methods to evaluate the signal. By combining frequency and peak analysis with logical triggers, successful seizure detection and on-demand systems can be constructed [67, 68].

Of course, the technical challenges with an implant capable of both detection and intervention are enormous. First, there are issues with biocompatibility. Since the implant is to be chronically inserted into a sensitive area where there must not be too much scar tissue forming, it is important that the device is as small as possible and has material and surface structures that does not alert an immunological response. Nanofabrication techniques might contribute to solve this issue.

In some cases the viral vectors carrying the genetic code for the opsins need to be injected deep into the brain tissue. The insertion and retraction of the injection syringe cause trauma to the tissue, damaging neurons and blood vessels along its path. Recently it was suggested that an additional modality could be added to an optrode [69]. In addition to the possibility to guide light and perform electrical stimulation and recordings, a microfluidic channel could be added to the construct. This channel is suggested to be used for the initial injection of the vector or form a route for continuous administration of drugs in the vicinity of the optrode tip. This would remove the inconvenience of retracting the syringe and subsequently inserting the optrode. It is also discussed whether this channel could be biodegradable hence minimising the trauma to the brain [69].

Also, since the penetration of light in brain tissue is limited, the need of an efficient light source is obvious. A low power consumption combined with a good light spread is essential to achieve a long endurance along with a large therapeutic volume. Current work at this laboratory evaluates utilizing nano-leds for this application.

### **6.3 Optogenetics and ethics**

Already at the level of the therapeutic application of epilepsy, optogenetics poses ethical questions that need to be discussed. Implantation of an optrode in the brain tissue might alter the structure and properties of the brain. Also, stimulation with the optrode will change the neural activity in the region, hence in theory affecting the very personality of the patient. So in addition to inheriting all the controversial aspects coupled to gene therapy, optogenetics also raise the question about how much of the human mind that it is acceptable to interfere with. As of today, we are certainly not able of reading thoughts, but there is not much stopping us from silencing them.

Gene therapy poses numerous ethical questions. A viral infection is irreversible; once the virus has infected the cells there is no way to remove the new gene. Also, what kind of genes is it acceptable to implant? A patient lacking a gene for producing a vital protein would greatly benefit from having gene therapy and people suffering from epilepsy might in the future be helped by optogenetics, but is it okay to treat healthy persons with genes coding for exogenous functions? It is necessary to debate these questions openly before releasing these powerful tools on any market.

Many novel medical treatments today need to be tested in animals, both in the developmental stage and for examining toxicity. The regulations for animals experiments are strict and focus on what is called the three R's. Refine, reduce and replace. *Refinement* is about decreasing the amount of suffering by optimising the experiment. *Reducing* means that the amount of animals involved in a study must be at a minimum. Finally, *replace* strive to find other ways, not involving animals, to perform the experiment [70]. In the case CNS of related research, there are few replacements for animals. Such replacement must mimic the complexity of the interconnectivity of neural tissue and that would require reconstructing an entire brain, or at least equivalent circuits. Though, recent progress in growing neurological tissue might contribute to solve the problem [71].

## 6.4 Future applications of optogenetics

Optogenetics shows great potential to become a new revolutionizing technique in bioengineering. It reveals a complete new way of interacting with tissue, adding optical light to the arsenal of how to manipulate cells.

Although most applications of optogenetics now are found in neurological tissue it is not farfetched to see it implemented in other cell types as well, considering the emerging OptoXRs. Imagine cells producing insulin, maybe contained in an implant with semipermeable membranes that release their product upon light stimulation. Then it is not unlikely that a closed loop system can be implemented which measures the blood glucose levels in real time and adjust the light stimulation frequency or intensity inside the implant in respect to the insulin need.

Another possibility is applications within neuroprosthetics. Imagine a cochlear-implant that, instead of sending vibrations to the hair cells, emits a light pattern that relates to the sound signal received by an external microphone. If the hair-cells can be modified to express for example channelrhodopsin, the light would activate frequency specific neurons with a higher spatial resolution than top of the line cochlear implants of today. The use of infrared light to excite neurons by heat in a similar manner is currently evaluated [72].

Much effort is put into combining optogenetics with two-photon (2P) excitation. Since 2P excitation, compared to 1P excitation, has the advantage of exciting only the focal plane, it is suggested that the technique could be used to target subcellular structures like dendrites and axons in neurons. Also, because of the longer wavelengths used, penetration deeper into scattering tissue is possible [73]. It might be possible that this method could be utilized in the effort of making communicating contact with a single synapse *in-vivo*. Construction of such device would require the cooperation of many disciplines, integrating many fields of technology with medicine and ethics.

There is an obvious disadvantage using light as basis for cell stimulation *in-vivo*. Light interacts heavily with biomolecules and hence does not propagate far in the tissue. In order to induce a large therapeutic volume it might be necessary to utilize multiple light sources which in turn will raise the demands on biocompatibility and surgical procedures upon insertion. An alternative could be to increase the intensity of one single light source and optimize its scattering. Though, there is a limit of

how much light the cells can tolerate in the immediate vicinity of such source without being subjected to damage [61].

To circumvent these problems, one could wish for an external source of stimuli that does not interact as strongly with tissue and therefore is not absorbed to the same extent as light. A radio-frequency (RF) magnetic field is known to heat nano particles consisting of manganese ferrite ( $\text{MnFe}_2\text{O}_4$ ). It has been shown that, if coupled to the cell membrane, these particles can open temperature sensitive ion channels (TRPV1) when activated by the oscillating field [74]. The raised temperature is restricted to the cell membrane and can be induced within seconds of RF activation. Similarly to optogenetics, gene therapy has to be used to introduce the temperature sensitive channel with high cell type specificity *in-vivo*. Spatial restriction could be achieved by a stereotactical injection. Also, the nanoparticles need to be administered so that they ideally only bind to the cells of interest.

## 7. Conclusion and future perspectives

In this study, transgenic mice were stimulated with an intrahippocampal optrode to produce afterdischarges that resemble TLE seizures. The occurrence of electrographical events was detected in order to characterize the model. Two types of events were found to occur; spontaneous events and afterdischarges. The spontaneous events were not related to the stimulations but rather reflected the level of sedation in the mice. Afterdischarges, on the other hand, increased with the number of stimulations. The finding that there is a kindling effect in the optogenetically stimulated animals stands in contrast to what is previously reported.

A second aim of the thesis was to investigate whether isoflurane or ketamine/xylazine affects the optogenetic seizure model. Afterdischarges were found to occur in the group anaesthetised with isoflurane but not in the ketamine group. Otherwise there was no difference, considering the tissue response, when comparing the two methods. A future, more thorough study of the effect of anaesthesia in optogenetic applications should include groups with varying doses of the different drugs. In the case of inhaled anaesthetics, it is hard to estimate the concentration in the blood. Though, it might be possible to monitor the level of sedation with help of a real time calculation of the BSR. Similarly for ketamine, a mathematical model of the metabolism could be utilized to calculate the drug concentration in the blood. The results of such experiments could be used to optimise the anaesthesia in many types of experiments utilizing optogenetics.

The final aim of this thesis was to refine the procedure in respect to anaesthetic method and optrode position. At the dose used in these experiments, ketamine/xylazine proved unsuitable for the optogenetic seizure model. Firstly, it blocks the afterdischarges and prevents the desired epileptiform activity to occur. Secondly, the heavy and fast breathing in ketamine anaesthetised mice overshadows the neural activity of interest. It might be that a lower dose would not have equal impact on the afterdischarges. Such a study, comparing different dose levels of ketamine/xylazine, could be of essence. Though, when reviewing the result from this study, the use of isoflurane instead of ketamine/xylazine is recommended.

There is more work to be done, considering the optrode position. The coordinates used could be evaluated by comparing the histological analysis with a brain atlas. Furthermore, other positions than the CA1 region could be considered in order induce afterdischarges more effectively.

A complicated but interesting experiment would be to stimulate conscious animals. No anaesthesia will then interfere with the optogenetic stimulations and there might be more afterdischarges developing. Such procedure would require a protocol much alike the one used in electrical kindling; the animal must be able to move independently of the cords and optic fibres attached to the implanted optrode. Also, similarly to electrical kindling, the optrode will have to be implanted on the animal well before the stimulations.

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