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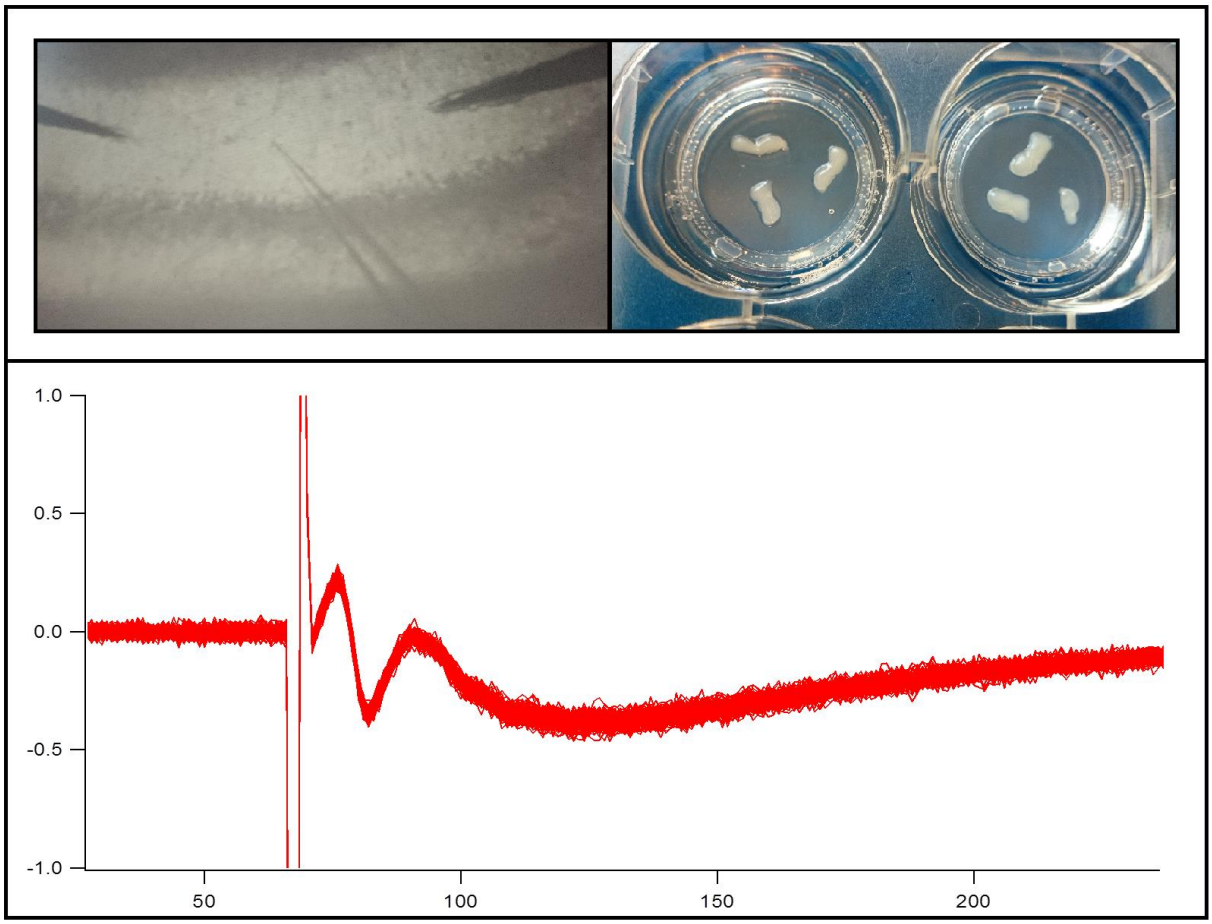

Organotypic rat slices cultured in human CSF

-An evaluation using extracellular field recordings

Master's thesis in Learning and Leadership

Andreas Gustafsson

CHALMERS UNIVERSITY OF TECHNOLOGY
In collaboration with
Department of Neuroscience and Physiology
UNIVERSITY OF GOTHENBURG
Gothenburg, Sweden 2014

Master's thesis 2016:149

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Abstract

This thesis present an investigation of a method for culturing hippocampal slices from rat in human cerebrospinal fluid. Slices were placed on Millipore™ membranes in wells cointaining human cerebrospinal fluid and incubated for 2-5 days. Control trials were made using Neurobasal-A as culture medium. The trials were evaluated using extracellular field recordings. In addition, pH and osmolarity in the culture media was monitored and used to adjust the culturing process.

The study shows that preserving acute-like properties of cultured slices for a prolonged time is possible, but that the culturing method is not robust. Acute-like recordings from cultured slices were obtained, and slice properties were shown to be preserved and recorded over several days from the same culture well, but the successful trials were few. Overall performance of the culturing was found to be poor with many slices degenerating in the culturing process. The study points towards early problems during culturing, which needs to be followed up. Some possible future research topics for the field of organotypic slice culturing are discussed and suggested based on the findings of the study.

In addition of the presentation of the research conducted, the thesis also covers a work conducted in connecting neuroscience and didactics. This was done in the form of teaching material on recent discoveries in neuroscience and their possible use for teaching methods. The material was attended for, and used in a course on learning theories given at the MPLOL master's programme at Chalmers University of Technology.

Keywords: Brain tissue culturing, human cerebrospinal fluid, electrophysiology, neuroscience, didactics.

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Glossary and abbreviations

aCSF	-artificial cerebrospinal fluid
AMPA	alpha-amino-3-hydroxy-5-methylisoxazole-4-proprionic acid, an agonist of the $AMPA_A$ and $AMPA_B$ receptors r
CA	Cornu Ammonis
DG	dentate gyrus, a part of hippocampus
EC	entorhinal cortex,
EPSP	excitatory postsynaptic potential
hCSF	human cerebrospinal fluid
LTD	long term depression, a long term down regulation of synaptic strength
LTP	long term potentiation, a long term up regulation of ysnaptic strength
NB	Neurobasal A
NMDA	n-methyl-D-aspartate, agonist till NMDA-recptorer
STD	short term depression, a biref down-regulation of synaptic strength
STP	short term potentiation, a brief up-regulation of synaptic strength
Volley	A term for the voltage gated channel deplarization response when stimulating the axons during extracellular field recordings

Introduction

This chapter covers the aim of the project, the research questions posed and the scope and the limitations of the project.

Purpose

The aim of the the research conducted in this master's project was to investigate and evaluate cultivation of rat hippocampal slices in human cerebrospinal fluid (hCSF) with the ultimate goal of answering the question:

> Are hippocampal slices from rat cultured in hCSF viable as a model for future research on human brain functions?

The investigation is of interest because; No reliable method for culturing hippocampal slices in hCSF exist today; Cultivation in hCSF would offer conditions that are closer to in vivo conditions than artificial CSF (aCSF) do; Having a reliable culturing method would open up possibilities for new research in neuroscience, such as investigation of neuronal network property changes when exposed to hCSF from patients with neurodegenerative diseases (Alzheimer's, MS etc). In addition to this, there was also a secondary purpose of using the acquired neurological knowledge to produce literature to be used in teaching of neurodidactical learning models, connecting the fields of neuroscience and didactics.

Background

In neuroscience, animal models are frequently used to discern the etiology of a certain pathology or disease, as well as to study the potential negative or positive impact of certain substances, endogenous and exogenous, on the neural development both on cell and neuronal network level. For this purpose, animals are raised in labs and exposed to certain treatments while examining the behavior, or subsequently killed and examined under the microscope and with advanced measuring tools such as electrophysiology. The breeding of animals poses both practical and ethical problems at times, and it is desirable to reduce the amount of animal trials used in the research. If scientists would be able to grow the desired tissue in vitro instead, and still obtain the same kind of data as from live animals, it could greatly reduce the need of research animals.

Cell cultures offer an excellent tool for this on the individual neuron scale. Neurons in cell cultures form spontaneous networks and are highly interesting for experiments such as the training of cultured human neurons to operate a robot by Pizzi et al (2009). However, the networks that form do not resemble those found in vivo. As of today, it is not possible to grow a working brain or part of a brain in vitro, so researchers have to resort to other methods. One frequently used option for research that cannot be conducted in live animals or humans is to use sliced tissue. In some cases it is desirable to keep the tissue alive for a long time. Thus, research on how to best grow various kinds of neurological tissue is conducted in several laboratories around the world.

Stoppini et al (1991) were among the first to describe a culturing method where sliced parts of hippocampus from rodents was cultured on permeable membranes partially submerged in media for several days. The neuronal functionality with respect to extracellular field recordings and tissue morphology was shown to be preserved and neuronal development continued in an in-vivo like manner. Subsequent trials by Gambrill and Barria (2011) and Carmeli et al (2013) have yielded similar results, and some experimentation has been conducted on different artificial culture media.

However, the sample sizes of the studies are in general small and there has been few and far in between studies published utilizing the method. It could be speculated that the membrane culturing method might not be as robust as the published studies indicate even for artificial media.

It is of a substantial interest for research on neurological diseases, such as MS and Alzheimer‘s disease, to be able to culture the slices at as in vivo-like conditions as possible. One target of investigation is whether cerebrospinal fluid, obtained from healthy volunteers compared to normal minimal growth media, does provide a more physiological condition while retaining neuronal survivability and normal function. No studies with long-term exposure to cerebrospinal fluid have so far been conducted on the subject.

This frontline neurological research could also prove to be of high interest from a didactic viewpoint. It is only recently that the importance of neurobiology for cognitive processes has begun to emerge as a field in education and future applications of today’s research could have a large impact methods for how to best teach different subjects to students, both in school and in work life.

Limitations in the scope of the project

The project was focused mainly on culturing slices of rat hippocampi in hCSF, and to some lesser extent in a Neurobasal-A ™ based aCSF. Efforts were made to keep conditions as similar as possible to physiological conditions within the brain. Other factors, such as pH, osmolarity and CO2 concentration during cultivation was monitored and to some extent analyzed in order to optimize culturing conditions. As the main scope was to investigate the usefulness of hCSF for cultivation, there was little effort spent on finding the optimal range for other parameters, other than maintaining stable conditions at values obtained from other studies. The results of the culturing experiments were mainly evaluated using extracellular field recording.

The secondary objective of connecting teaching and neuroscience was attempted by preparing teaching material about neurodidactic models for learning. This was done in the form of a text covering some recent neurological discoveries connected to learning and providing a discussion task in the form of a case to be used during two subsequent lectures in the course *Analyzing Learning* (MVE380) given at Chalmers University of Technology for aspiring teachers on the masters programme in Learning and Leadership (MPLOL).

Introduction to the brain and neuroscience

This part covers the field of neuroscience more in depth. It is intended as an introduction to the field of neuroscience for those with a background in general science but no specific understanding of topic. Starting with the human brain and the use of rats as model organisms, it covers the very basics up to advanced parts needed to understand the work that was conducted in this project.

General outline of the human brain

The human brain is a huge and complex structure of neurons and other cells. It is divided in different parts. The major part of the brain is called the cerebrum and the smaller part at the back is named cerebellum. The folded surface of the cerebrum is called the cerebral cortex. The cerebral cortex is subdivided into the frontal lobe, parietal lobe, temporal lobe and occital lobe. This cortex connects to several parts located under it. One of these is the limbic system, connected to both the cortex and the cerebellum. The limbic system contains several sub-parts, including thalamus, hypothalamus and the hippocampus. The hippocampus is the part of special focus in this report.

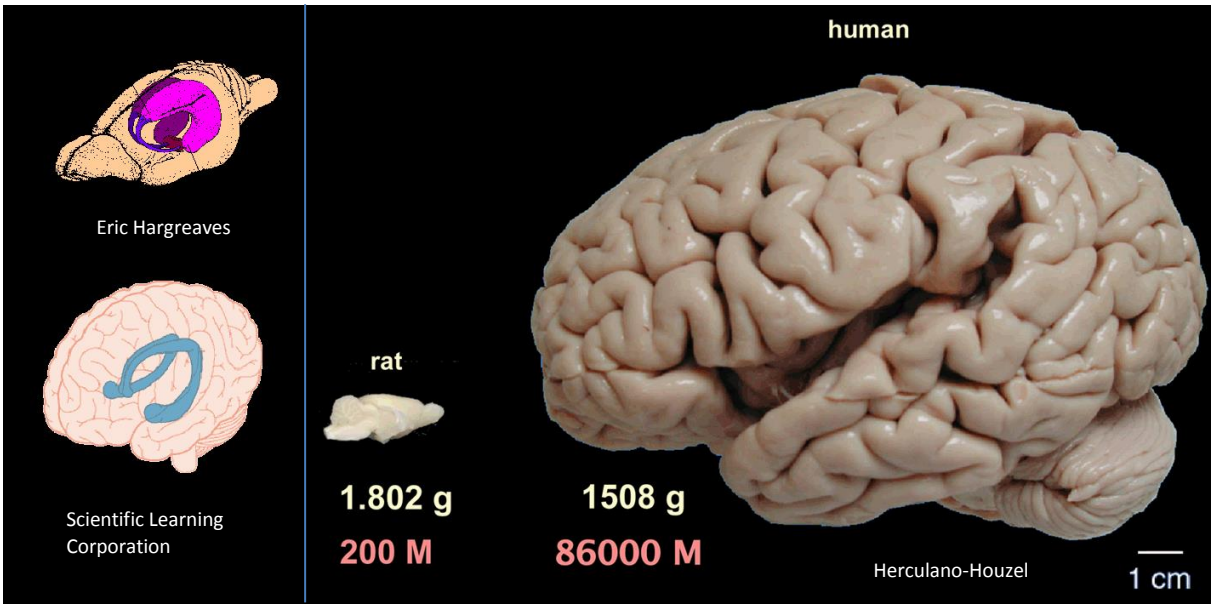

Figure 1. Left: The location of hippocampus in human (bottom, blue) and rat (top, purple). Right: The rat brain and human brain to scale with estimates on weight and neuron count. Pictures collated from http://hargreaves.swong.webfactional.com/seahorse.htm, http://www.BRainConnection.com, Herculano-Houzel (2009).

The rat as a model organism in neuroscience

Research in neuroscience is conducted on a variety of levels ranging from single molecule level and molecular interactions via single cell level (both in vitro and in vivo), tissue level (in vivo and in vitro) up to macro level of the whole organisms. The preferred level when possible is in-vivo investigations in humans, because it is the actual system one is interested in investigating. To be able to conduct research on this level without causing injuries or altering functions is rare, and for many investigations there is a need of alternative model systems. Cell cultures, tissue cultures and animal models are frequently used instead. These model systems allow more invasive research to be conducted.

Cell cultures are the least complicated model systems and frequently used to study single cells and interactions between neighboring cells. Other research questions need to be investigated in-vivo and for this animal models are used. Rodents, such as mouse and rat, are among the more popular ones. They are small and have short genereation times that allow faster research than larger animals, which makes keeping them easier, and being mammals, they share more properties with humans than fishes, reptiles or birds do. However, as they are small and not as closely related to us as other possible

research animals (such as monkeys and apes), and the brain sizes and structures differ considerably from human brains, there is always a need to be careful with the interpretation of the results. Do they generalize to humans? That is usually a hard question to answer and studies in model organisms need follow up studies testing if the effect observed is consistent in humans as well.

Rodents are also frequently used as a source for tissue to perform experiments on. In neuroscience it is common to perform experiments on acute (freshly cut) brain tissue from different parts of the brain. Less common, but emerging as methods improve, is experiments on cultured tissue. I.e. tissue that is cut freshly from live animals and cultured (i.e. preserved in-vitro) for a prolonged time. The work in this thesis was performed on hippocampal slices from rat.

The hippocampus

The hippocampus is a small, tubular, sea-horse shaped (hence the name), part of the brain located in the medial temporal lobe in both hemispheres. I.e. each half of the brain has a hippocampus. The hippocampus is present in all mammals. The function and cellular structure is highly conserved among species, gross morphology and the size of the hippocampus relative to the whole brain varies among species. It is among the most studied and well known parts of the brain both in model animals, such as rodents, and in humans.

Investigation of the temporal lobes in general and hippocampus role in specific for memory formation started with Scoville and Milner (1957), who had a patient with injured temporary lobes. Extensive research indicates that the hippocampus is vital for long term memory formation, though the mechanisms behind are not completely understood. The hippocampus has also been found to play an important role in spatial navigation. O'Keefe (1976) showed that specific neurons located in hippocampus of rats could be linked to the rat's location. Several studies have since confirmed and elaborated on the connection. Today several types of spatial-processing cells, such as head cells and grid cells (Hafting et al 2005) have been identified in the hippocampus and associated structures, such as the entorhinal cortex.

The cellular structure of the hippocampus is highly organized with neurons aligning in clearly defined layers, giving cross sections of it a typical cinnamon-roll like shape. The cell bodies of the pyramidal neurons that make up 90% of the neurons in the hippocampus can be detected as darker bands when visually inspecting a cross section. The hippocampus is connected to the Entorhinal Cortex (EC) located in the parahippocampal area, and consists of the Dentate Gyrus (DG), the Cornu Ammonis (CA) parted in CA1, CA2 and CA3 regions, and the subiculum (see Figure 3).

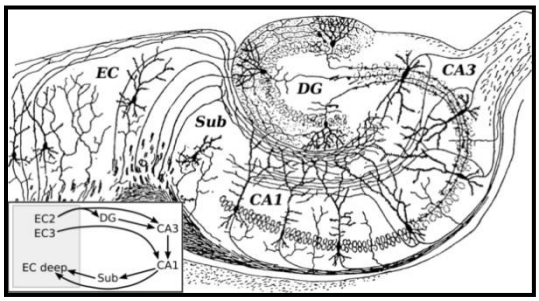

Figure 2. Top: Drawing of the hippocampus with a schematic of the trisynaptic pathway by S.R. Cajal(1911). The darker bands correspond to the cell bodies of the pyramidal neurons of the hippocampus and the extensions of the dendrites and axons from these are schematically represented. In the lower left corner an addition of a trisynaptic pathway flow-chart schematic has been made, artist unknown.

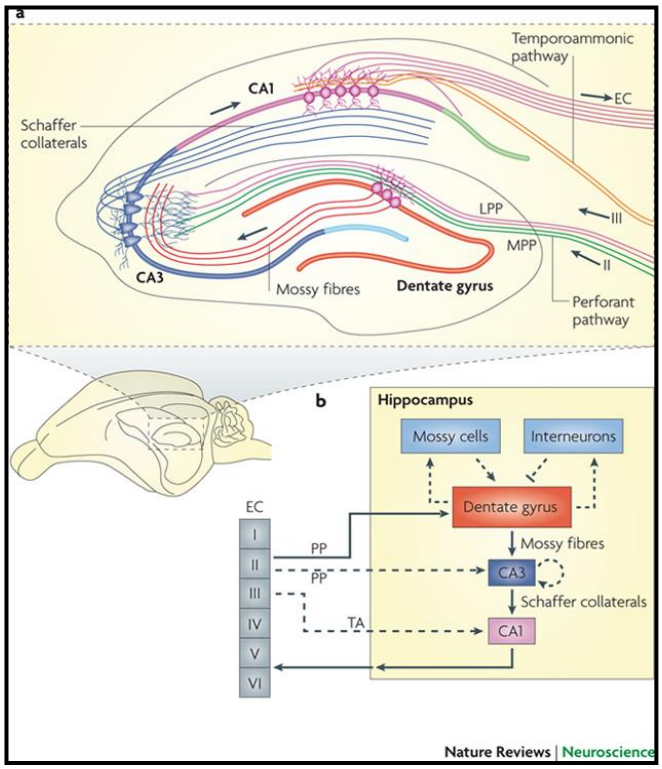

Figure 3. Right: Schematic of the hippocampal structureand it's location in the rat brain. Thin lines represent axons extending from the cell bodies. The trisynaptic pathway is shown in the lower flow chart (full arrows) along with other connections within the hippocampal structure (dotted arrows).Picture from Deng et al (2010).

The trisynaptic pathway

The major signaling pathway within the hippocampus is referred to as the trisynaptic pathway, named so because it has a distinct and prominent progression over three synaptic connections. Starting with input from Entorhinal Cortex (EC) pyramidal cells in layer II, these cells project their axons to the cells of the Dentate Gyrus (DG). The DG neurons in turn project their axons, called mossy fibers to the CA3 pyramidal cells. From CA3 axons called Schaffer Collaterals project to the CA1 pyramidal cells, that in turn projects their axons to the subiculum. The subiculum reconnects to the EC at layer V. The axons from CA1 also reach out to other parts of the medial temporal lobe structure.

Hippocampal slice cultivation

Extensive research on the hippocampus has been conducted by using slices, both cross sectional and longitudal. Common methods for this research has included; Staining the of slices for visualization of the neurns, beginning with the research of Cajal and Golgi in the late 19:th and early 20:th century (Bear et al 2007); Extracellular field recordings where the electrophysiological properties of neurons are investigated on a macro scale; Patch clamping, a method not covered by this report, in which electrophysiological properties of individual cells are investigated. Much research has been conducted on what is referred to as acute (freshly cut) slices, but, as mentioned in Background, there has been need of a method of keeping the neurons in the cut slices alive for extended periods without altering their properties.

The cells of the brain

The brain in mammals contains several different types of highly specialized cells. The two most common cell types are the neurons, which are cells that are responsible for most of the rapid intercellular communication in the nervous system and the brain, and the glia, that make up much of the supporting structure of the brain tissue. Azevedo et al (2009) estimate the ratio of neurons to glia cells in the human brain to be close to 1:1. This is for the brain as a whole, but the ratio varies considerably between different parts of the brain (Herculano-Houzel and lent, 2005).

In addition to these cells, the brain also contains several other cell types in smaller amounts. These include the cells that make up the blood vessels, membranes and other support structures, as well as highly specialized cells for other functions. One such type is the phagocytic microglia, which can be thought of as janitors of the brain. They eat cells and parts of cells that need to be removed to keep the brain healthy. The interaction of all of the cells in the brain has not been researched much today. Historically the research in neuroscience has been focused on the neurons, but recently there is an increasing interest in glia and other cell type interactions both with neurons and each other.

Neurons

The neurons (commonly termed nerve cells in popular science) are cells that are specialized in intercellular communication. This rapid communication is done chemically at specialized terminals called synapses. There exist several types of neurons with different roles. A few are sensory reacting on touch, light, chemicals or temperature, giving us our five senses, and others are used for communication with other cells in the body, for example sending motor signals to muscles. However, most neurons are located in the brain and in the central nervous system and communicate rapidly with other neurons.

It is commonly appreciated that the brain contains around 10^{11} neurons. With individual neurons forming from hundreds up to tens of thousands of connections to other neurons, the network of the human brain is quite extensive. As a comparison, the number of neurons in the rat brain has been estimated to around $2*10^{8}$ (Herculano-Houze and Lent 2005).

All neurons share a few common traits and differ in others according to their specializations. They have a central cell body, the *soma*, which contains the cell nucleus and most of the organelles of the cell. From the soma, one or several protrusions extend. They are broadly categorized in two groups. One is the axon, which is a long, quite thin, protrusion with a fairly uniform diameter all the way and has different membrane composition than the soma. It extends from the axon hillock close to the soma out to the axon terminal. Axons can be branched and end in several terminals. The axon is the main path along which signals are sent from the neuron to the cells it connects to. The axon terminal contains the presynaptic part of a synapse, i.e. the sending part.

The other group of protrusions is the dendrites, which are branching out from the soma in a tree-like manner connecting to the cells around the neuron. Some dendrites have synapses directly attached to the membrane while others have small protrusions called dendritic spines, each ending in a specialized synapse. These spines are the main area of input from cells communicating with the neuron.

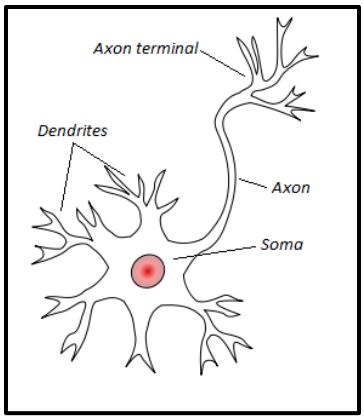

Figure 4: Schematic picture of a neuron showing the soma, dendrites, axon and axon terminal. At the end of the branches of the axon terminal, synapses are in contact with other cells, sending signals from the neuron. At the branches of the dendrites synapses are in contact with other cells receiving signals. Picture is cropped and modified from the original (wikimedia commons, Jonathan Haas)

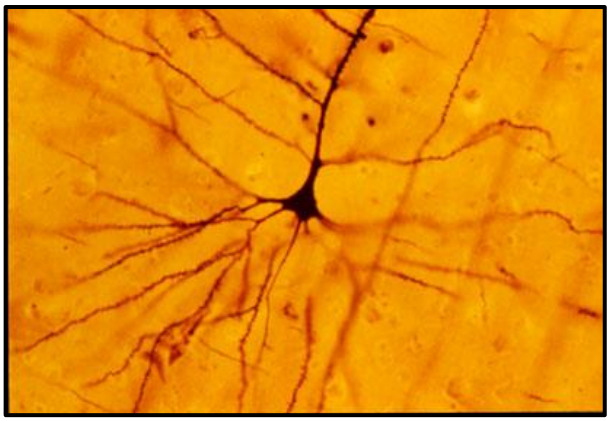

Figure 5: A Golgi stained pyramidal cell showing the pyramidal shape of the soma and the axon protruding from it to the top of the picture. From the bottom part, dendrites can be seen protruding from the pyramid shaped soma. Photo: Bob Jacobs, Laboratory of Quantitative Neuromorphology Department of Psychology Colorado College.

Since neurons come in many different specialized forms, it is common to classify them into different groups that fill different roles in the communication network of the brain. In neuroscience, the first distinction is usually made between excitatory and inhibitory neurons, i.e. they are classified based on function. Excitatory neurons often extend their axons far communicating with cells in other parts of the brain, while inhibitory neurons, commonly named inhibitory interneurons, typically connect to the local cell cluster regulating the signals from the excitatory cells in it.

Further classification is based on morphology. The neurons are divided in groups based on the shape of the soma, location of the soma relative to the axon, and the dendritic tree structure of the neuron. A sub-class of excitatory cells is the excitatory pyramidal cells, named after the pyramidal shape of the soma. These cells generally have long axons projecting far away from the soma and relatively few dendrites. The pyramidal cells make up an extensive part of the hippocampus neurons.

Glia

Glia are commonly thought of as the support cells of the neurons. Roughly equal in numbers to the neurons, they make up a considerable portion of the volume of the brain. They also come in several types with different shapes and functions. The most common type is the astrocytes, which fill up the space between the neurons of the brain. They are involved in regulation of the local concentrations of neurotransmitters outside the synaptic cleft, preventing signals from propagating too far from the synapse and affecting neighboring synapses. They also communicate with the neurons and have inhibitory and excitatory functions (Nedergard, 1994) (Parpura et al, 1994), though much about the astrocyte-astrocyte and the astrocyte-neuron interactions remains unknown today.

Another type of glia is the myelinating glia. These cells envelop the axons of some neurons with an insulating sheath called myelin that increases the propagation speed of the electrical signal in the axon.

Neuronal communication

Neurons communicate via electrochemical signals. Within a neuron the signal is transmitted fast via something called the action potential, which is a change in electrochemical potential along the axon caused by the local change of ion concentration very close to the membrane. Between cells, the communication is conducted chemically via signal molecules released and received at certain receptor rich terminals called synapses.

The membrane potential

The resting membrane potential is an electrochemical potential that exists between the inside of the cell and the surrounding environment. The cytosol (the fluid contained in the cell) contains many different ions at concentrations that are different from those outside the membrane. This concentration difference is possible because the membrane is semipermeable, i.e. some substances can pass across it while other cannot. Ions cannot pass through the cell membrane without the help of surface proteins. These proteins can be separated in two different groups. One is the ion pumps, that transport specific ions across the membrane in the opposite direction compared to the concentration gradient. I.e. the ions are pumped from lower concentration to higher, emptying the inside of the cell of a specific ion type, or transporting a specific ion into the cell to achieve a higher concentration than in the extracellular fluid. The sodium-potassium pump (NA^+/K^+-ATPase) is the prime example of an ion pump. It uses ATP (adenosine triphosphate) as the energy source to drive the pumping of sodium ions out of the cell and potassium ions into the cell (Bear et al, 2007).

The other type is the channel proteins that allow ions to pass through the membrane in the direction of the concentration gradient, i.e. ions flow from higher concentration to lower. Most of these are gated channels that are closed and opened by certain triggers, but some are constantly open and allow for a trickle of ions to diffuse back into the cell after getting transported out. The pump rate and the trickle back rate for each ion reach an equilibrium and the concentration difference between the inside and the outside of the membrane of these ions gives rise to a potential difference for the specific ion. The total membrane potential is the sum of all these partial potentials contributed from each ion type and the total equilibrium is called the resting membrane potential. Under normal conditions it lies close to -70mV in human (and other mammal) neurons. Commonly, the partial contribution from individual ion types is calculated (i.e. modelled mathematically) using the Nernst equation or the Goldman equation (Bear et al, 2007).

Gated ion channels

Not all channel proteins allow ion passage all the time. Some channels are gated and only open under certain conditions. Two common types active in neuronal signaling are ligand gated channels and voltage gated channels.

Ligand gated channels are channels that open or close when certain ligands, small signal molecules bind to them. This is either caused by direct blocking of a channel by the ligand (sterical inhibition) or by the ligand binding to some part of the protein complex causing structural changes opening or closing the channel. Depending on the chemical structure of the binding site, the channel can be more or less specific to its ligands. Some channels are highly specific and (almost) only bind one ligand, while others can be affected by a broader range of chemically similar ligands. Ligand gated channels that open on binding a ligand are typically found at the receiving end (postsynaptic) of a synapse. The opening of the channel causes the concentration gradient of the ions that the channel is permeable to, to drive a net flux of those ions across the cell membrane. The direction of the flow depends on the electrochemical gradient. I.e. is dependent on both the concentration gradient for the ions the channel

is open to and the voltage gradient over the membrane. The ion flux changes the membrane potential locally around the channel.

In the case of a depolarization, the local membrane potential around at the synapse goes from negative to positive for a brief moment before the ion pumps are able to restore the initial equilibrium concentration. This brief potential change is referred to as local depolarization of the membrane and lasts for a few milliseconds.

Another type of ion channel is voltage gated channel. These channels are located along the axon shaft and along some dendrites. Instead of needing a ligand to bind to open the channel, a voltage gated channel changes conformation when the membrane potential close to it changes. At a certain potential threshold, the channel opens. This allows ions to flow along the electrochemical gradient into or out of the cell. The most common voltage gated ion channels are specific to sodium (Na^+), potassium (K^+) and/or calcium (Ca^{2+}) ions.

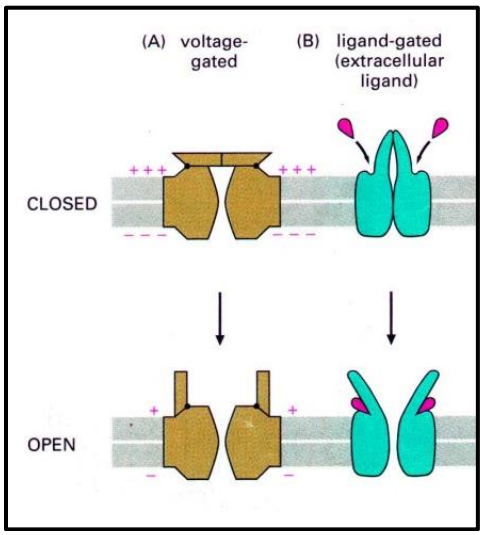

Figure 6. Schematic picture over voltage-gated and ligand-gated ion channels.A voltage gated ion channel changes configuration when the local membrane potential is changed and lets through an ion flow. Ligand gated channels change configuration and opens when a ligand binds in to them.Picture from Austin Pey University, artist unknown.

The action potential

The action potential is a fast propagating change in local potential along an axon. The action potential itself starts at the axon hillock (the initial segment of the axon) close to the soma and travels out to the axon terminal(s), but in order for it to start; the neuron needs to get input from the synapses on the dendrites. This input starts with the opening of ligand gated ion channels at the synapses on the dendrites of the neuron. Every change in polarization at the synapses contributes a small amount to the membrane potential at the voltage gated channels located at the axon hillock. Spatially closer changes contribute more than more distant ones. This is reffered to as spatial summation of the potential.

Another factor affecting the opening of voltage gated channels is temporal summation. This is the effect of the same ion channel opening several times in a short time interval. If the channel opens a second time before the ion pumps in the cell membrane are able to restore the membrane potential, there will be a lingering potential change with each opening, pushing the potential closer and closer to the activation threshold of a voltage gated channel.

The total effect of the temporal and spatial summation is that a strong stimulance of several synapses close by with many ion channels opening can cause an immediate opening of a voltage gated channel and a rapid signal response, while a smaller stimulation occurring many times will eventually build up to the opening of the channels.

When the local membrane depolarization changes are large enough, either from several synapses simultaneously, or from a few synapses over a longer period, the change in potential at the axon hillock will be large enough to trigger the opening of the sodium/potassium channels located there and this in turn will cause the neighboring channels downstream the axon to open. This in turn will open the next channels and leads to a self-propagating wave of local depolarization moving along the axon. This depolarization wave is called an action potential and cuses a measureable electromagnetic field.

In the generation of action potentials in the excitatory cell communication in neurons, the voltage gated sodium channel is the most prominent one. After the first voltage gated channel activation, the action potential becomes self- propagating as the depolarization of one voltage gated sodium channel causes the depolarization of the next. The inflow of sodium ions will continue a bit past where the charge over the membrane is zero, making the potential positive for a brief time before the voltage gated sodium channel close and the sodium-potassium pump can restore the local resting potential of the membrane (see Figure 7). For a brief time after closing, the channel cannot open again. This time is called refractory period. That there is a refractory period for the sodium channel is important, since it prevents the back-propagation of the signal up to the axon hillock. This ensures that the axon signal only travels one way, sending information to the axon terminal.

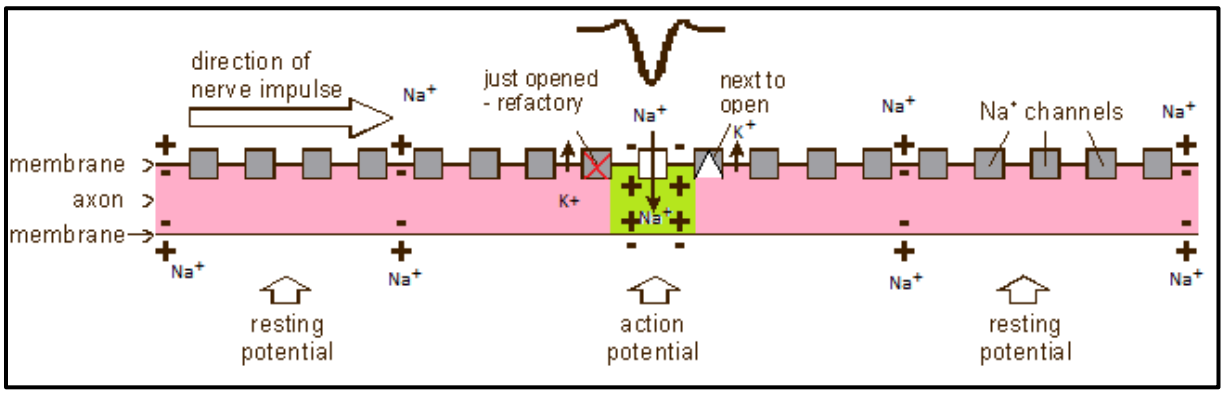

Figure 7: Schematic of the propagation of an action potential. As the action potential moves, it causes the voltage gated sodium channels to open, which causes an inflow of sodium ions into the axon. At the same time, potassium ions leave the axon in areas close to the action potential via potassium channels. This causes the typical waveform with small increases in potential at with larger dip between them observed as the volley in extracellular field recordings.

The synapses

The synapse is the site where intercellular communication takes place. A synapse consists of the presynaptic terminal on the signal sending neuron, a postsynaptic terminal on the signal receiving neuron. Between these terminals, the synaptic cleft, a gap of 20-40 nm length between the synaptic terminals, is located. At the presynaptic side of the cleft, the neuron contains small vesicles containing neurotransmitters. Neurotransmitters are a diverse group of small molecules that have high affinity for receptors located at the post-synaptic side of the synapse. These receptors occur in large numbers and the composition of receptor types and their numbers vary from synapse to synapse.

When an action potential has propagated along the axon of a neuron and reaches the axon terminal, a complex signal cascade at the presynaptic side of the synapse occurs, which causes the vesicles containing neurotransmitters to fuse with the cell wall and release their contents into the synaptic cleft. The neurotransmitters then diffuse over the cleft in milliseconds and bind to specific receptor proteins at the post-synaptic side, which in turn trigger a signal cascade. The nature of this cascade depends on the type of receptor activated and on the amount of receptors that activate synchronously. Ionotropic receptors are ion channels that open and either increase (excitatory synapses) or decrease (inhibitory

synapses) the chance that an action potential will fire in the receiving neuron. A depolarization increases the chance of action potentials and a hyperpolarization decreases the chance. Metabotropic receptors on the other hand are receptors that are not ion channels and instead give rise to other signal cascades that can have a wide range of effects.

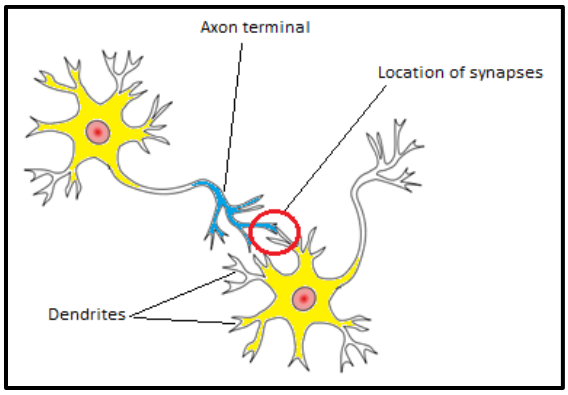

Figure 8. Schematic picture of the location of the synapses.One connection is shown. In reality each neuron forms from hundreds to several thousand connections to several neighbouring cells. The synapses are the relay stations between the axon terminal (highlighted in blue) of one neuron and a dendrite of a neighboring cell (dendrites and the soma highlighted in yellow).Signals are sent from the axon hillock of one neuron to the dendrites of the next.

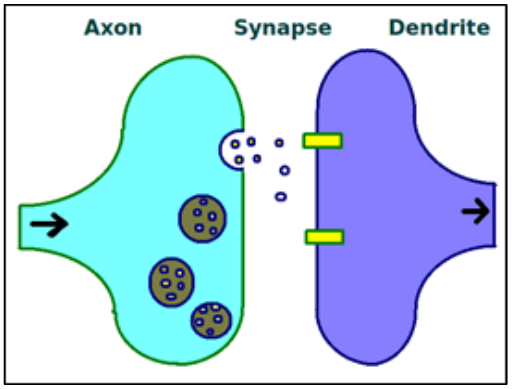

Figure 9. Schematic over a synapse. The left side is the pre-synaptic termina and the right side the post-synaptic terminal. Here vesicles with neurotransmitters are located and fuse with the cell wall to release them when an action potential from the axon reaches the terminal.The neurotrasmitters diffuse over the synaptic cleft (the gap between the terminals) to the post-synaptic terminal. There the neurotransmitters bind to receptors (symbolised by yellow rectangles here). If the recptor is an ion channel it opens and the membrane depolarizes. Original picture from Wikimedia commons. The picture has been edited.

Receptors

There exist several types of receptor proteins that are more or less specific to their respective signal substances. Receptors can be either excitatory or inhibitory. An excitatory receptor increases the chance that an action potential will be fired when a neurotransmitter binds to it and an inhibitory receptor decreases the chance of an action potential.

In addition to this, the receptors can be classified as having either ionotropic effect or metabotropic effect. Ionotropic receptors are ligand gated ion channels that give fast responses to activation while metabotropic receptors function via second messenger pathways that can be much slower.

The most common neurotransmitter for excitatory synapses is the amino acid glutamate, which interacts with the AMPA-receptor-, the NMDA receptor- and the Kainate receptor families. One of the most common inhibitory neurotransmitter is GABA (γ-Aminobutyric acid).

Figure 10: Chemical structure of the neurotransmitter glutamate (left) and GABA (right).

Excitatory and inhibitory synapses
Synapses are either of excitatory or inhibitory type, containing a majority of either inhibitory or excitatory receptors. An excitatory synapse increases the chance to start an action potential in the signal receiving neuron when they receive signal molecules, while inhibitory synapses decreases the chance of an action potential. What determines if a synapse is excitatory or inhibitory, is the combination of neurotransmitters released at the pre synaptic side and the type of receptors located at the post synaptic side.

AMPA receptors
AMPA receptors are a family ligand gated on channels that have glutamate as the activating agonist. The name is derived from AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid), an artificial agonist for the receptor family. They are ligand-gated tetrameric receptors consisting of a total of four subunits, picked from four possible subunits. (GluR1, GluR2, GluR3, GluR4).
AMPA receptors are quick to activate and permeable only by sodium and potassium. This allows a net influx of sodium to the cell interior and a net outflow of potassium along the electrochemical gradient when activated. This causes a depolarisation of the cell membrane close to the receptor pushing the potential closer to the activation potential of voltage gated channels. I.e. AMPA receptors are excitatory.

NMDA receptors
The NMDA receptor family is a group of excitatory glutamate receptors that are both ligand and voltage gated. The ligand gating requires L-glycine as a co-activator in addition of glutamate (Johnson and Ascher, 1987). The ion channel is also blocked by extracellular magnesium at the normal resting potential of the cell membrane (Nowak et al 1984) but not at more depolarized potentials. Thus the receptor will only activate and the ion channel be opened if it is simultaneously activated by both glutamate in the presence of L-glycine and the cell membrane around it is locally depolarised.

NMDA receptors involved in synaptic plasticity are calcium (Ca^{2+}) permeable, and activation of the recptors leads to an inflow of calcium ions into the cell. As calcium ions interact in many ways in the neuron, the influx of calcium from these receptors is involved in both ionotropic and metabotropic responses to NMDA activation.

GABA receptors
GABA receptors are a wide class of inhibitory receptors which GABA is an agonist for. There exist both ionotropic $GABA_A$ and metabotropic $GABA_B$ receptors. The ionotropic GABA receptors work by opening ion channels that increase the polarization of the cell membrane. This decreases the chance that an action potential will start in the neuron.

Picrotoxin (PTX) is a neurotoxin occurring naturally in the plant *Anamirta cocculus*. It is an inhibitor of $GABA_A$–activated chloride (Cl^-) channels. This prevents the inhibiting effect of the $GABA_A$–receptor and increases the chance that an action potential will occur in the neuron affected. PTX frequently used in neurophysiological recordings in order to make neurons more likely to fire action potentials, increasing the chance of detecting neuronal activity over noise level.

Synaptic strength
The strength of a single synapses signal transmission is dependent on several factors. On the pre synaptic side, it is affected by the amount of potential release sites that are present and the probability of release of neurotransmitters at each of these. Every action potential will not release neurotransmitters into the synaptic cleft. At the post synaptic side, the response strength is dependent on the amount of ionotropic receptors that are present and on the current membrane polarization state.

Synaptic plasticity

Synapse response may change over time. It includes both very rapid changes in synaptic strength lasting from milliseconds to seconds and more long-term effects lasting from minutes to years.

An increase in the synapses strength is called a potentiation and decrease is called a depression. Synapse regulation can occur both at the pre-synaptic and post-synaptic side of the synaptic cleft. Transient changes that last from milliseconds to minutes (short term potentiation and depression) are regulated in part by pre-synaptic mechanisms, that affect release probability of neurotransmitters and availability of vesicles containing them (Zucker and Regehr, 2002).

More prolonged changes in synaptic strength also occur. A long term increase is called Long Term Potentiation (LTP) and can last from hours up to years. Correspondingly, a long term decrease is called Long Term Depression (LTD). These long-term changes are mainly due to post synaptic alterations.

Several mechanisms for the cause of LTP has been proposed and none of them has to date been able to fully explain the complex process of synapse strengthening. One proposed mechanism is that calcium permeable NMDA receptors are necessary for an LTP (Poncer et al, 2002). The inflow of calcium triggers an increase in incorporation of AMPA receptors in the synapse, almost immediately increasing the synaptic strength. This local increase in synaptic strength does not affect neighboring synapses and is referred to as Hebbian plasticity (in contrast to homeostatic plasticity, which is a total up- or down regulation of synaptic response strength in the whole neuron). The calcium permeable NMDA receptors only activate if there is both pre- and post-synaptic activity at the same time, i.e. if there is repeated signaling in a short time span. Thus, in order to activate an LTP response, a repeated rapid burst signal is often used as this ensures synchronous activity of both synapse terminals. Another mechanism proposed for LTP causation involves increased synthesis of AMPA receptor proteins and takes longer time and might affect a whole dendrite or even the whole neuron.

The Hebbian plasticity regulation of LTP and LTD responses to stimulation is thought to be an integral part of memory formation. Specific memories are thought to be stored in circuits of neurons. These circuits are called *engrams* (a term originally coined by Richard Semon as early as 1921). While the mechanisms of the encoding are not fully understood, it has been proposed that the LTD and LTP regulation within the circuit are integral parts of the memory formation and storage (Poo et al 2*016).*

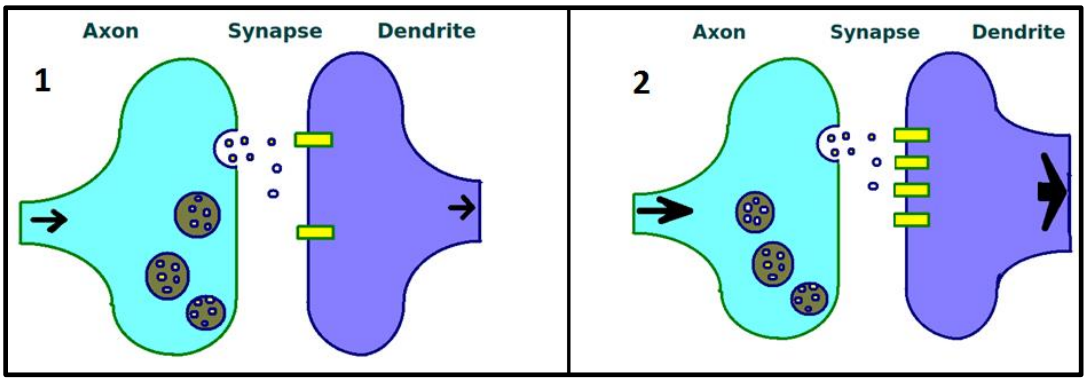

Figure 11. Schematic over long term potentiation (LTP) of a synapse.Left side shows the synapse before potentiation. The number of ionotropic receptors is low and the resulting depolarization from each receptor is low, requiring a longer time of stimulation to trigger a propagating action potential.The right side shows the same synapse after potentiation.More receptors are present resulting in a stronger depolarization from thed same amount of neurotransmitters.Original pictures from Wikimedia commons.The pictures have been edited.

Electrophysiology and field recordings

The voltage gated sodium channels in the axon of a pyramidal neuron can be activated by any depolarization event and is not dependent on the naturally occurring depolarization at synapses. This is exploited in electrophysiology, where several methods involve exogenous stimulation of voltage gated channels to cause them to open. By stimulating any individual axon with a high enough current, an artificially induced action potential will occur and propagate down the axon towards the terminal. At the terminal it will trigger the release of neurotransmitters. If enough synapses at the receiving end activate simultaneously, the induced action potential will give rise to a responding action potential in the cell.

Fields as the sum of all electrical potentials at the site of recording

This mechanism is used in the electrophysiological method called extracellular field recording. Slices of neuronal tissue are places in a recording well and held in place with a *grid* (a small curved metal piece with very thin nylon strands tied to it). The well is filled with an oxygen bubbled perfusion bath supplying the cells of the slice with nutrition and oxygen. By placing a wolfram coated stimulation electrode in the tissue along axons, a response may be recorded from cells located one synaptic cleft away from the stimulation. In order to obtain recordings above noise level, several axons needs to be stimulated, and they need to connect to enough synapses responding with an excitatory post synaptic potential (EPSP). The excitatory post synaptic potential is the potential change caused by the opening of the ion channels at the synapse; i.e. the measured resulting potential change from the depolarization of the cell membrane at the synapse.

What is recorded is the sum of all individual synaptic responses at the recording electrode. Each synapse will contribute with a share that is too small to be recorded at any one time, but if enough neurons activate enough synapses responding with an EPSP, the sum of them pass the threshold for detection. When stimulating hippocampal slices, it is common to use thin slices of tissue and the electrodes are typically placed in the well defined regions of the hippocampus, such as CA1, CA3 or DG. In this study, the stimulation electrodes were placed in stratum radiatum in order to stimulate the Schaffer collaterals extending from CA3 to CA1 (see Figure 3**Error! Bookmark not defined.**).

Common practice when conducting extracellular field recordings is to record a baseline of responses to single pulses at a frequency of 0.1-0.2 Hz for a period of 10 min up to 1h, followed by an LTP-inducing protocol of several fast bursts, and then a longer recording period with the same frequency as the baseline. This results in recording files containing wave data for hundreds of individual recordings from the same slice.

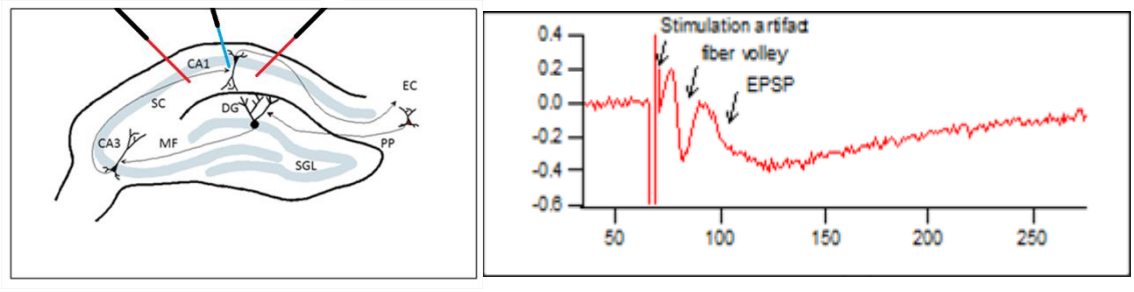

*Figure 12: **Left**: Electrode placement when recording with dual stimulation electrodes from the CA1 area of hippocampal slices of rat. **Right:** Typical example of a single sweep from a rat of 15 days age recorded with this electrode placement. The first big dip in the curve is the fiber volley, which is the measured effect from the axon depolarization in the stimulated cells. The second is the EPSP response. The 0 potential corresponds to earth. The scale on the bottom axis is recorded data points. 10 points correspond to 1 ms.*

Stimulation effect

When stimulating, the equipment usually picks up a strong disturbing initial signal. This artifact is transient. The cause of it is the conduction of electrical current in the perfusion bath and the resulting field potential. The artifact is present even if the electrodes are placed in the bath without a slice to record from.

A few milliseconds after the artifact, there is a slight increase in potential, followed by a large dip and a recovery up to the baseline. The shape can be seen in Figure 12. This first dip corresponds to the measured depolarization along the axon and is termed volley, as it comes as a direct response to the stimulation. The magnitude of the volley depends on how many axons were stimulated and is in general a better measurement of the actual stimulation strength the neurons received than the absolute numbers in current. This is because the resistance is not uniform within the slice and electrode placement and stimulation depth affects the resistance. The volley will propagate from the stimulation site in both directions along the axon. This happens because unlike in naturally occurring action potentials in the axon, there are no voltage gated sodium channels that just opened and are in their refractory state where they cannot open again, and thus nothing prevents the propagation of the volley in both directions. This means that there is no need to place a stimulation electrode upstream of the recording electrode along the axonal pathway.

After the volley, there is a second dip that sometimes overlaps partially with the volley. This corresponds to the sum of all synapse responses in all neurons close enough to the recording electrode. This response is called an EPSP (Excitatory Postsynaptic Potential) and is located directly at the synapse. The total EPSP recorded is dependent on the number of synapses stimulated, and on the synaptic response strength. Typically, in young animals <7 days, there are few and weak synapses, and it is difficult to achieve a response at all. As the animals grow and the synapses (and the spines they are located on) mature, the EPSP response gets stronger and stronger for the same initial stimulation. Typical for a rat of age of 10 days is an EPSP of about the magnitude of the volley and at age 25 roughly 4 times the magnitude of the volley.

The initial change (i.e. the time derivative of the local potential at the recording site) both in volley and field as it drops is linear with respect to time. The linear change continues for a brief moment, when all synapses activated have open ion channels, after which the linearity is lost as individual synapses at the activated neurons have closed channels and are repolarizing while others may still be depolarizing. The slopes of the linear regions of the curve (for the volley and the EPSP), which is the time derivative of the volley and the EPSP, is usually what is used when comparing fields from different trials with each other. Other measures that are sometimes used are the volley and EPSP peak magnitude, and the area between the curve and the resting potential (that is the integral of the volley and EPSP values). For an ideal signal, the area would be a better measurement, but since each trial contains several sweeps recorded and there is usually overlap between increasing and decreasing potential between the fields, and there may be early action potentials fired in the receiving neurons influencing the potential at the recording site; the average area is not as accurate as the linear regions where the line slope measurement is taken.

LTP

Synaptic plasticity is the key to long term changes in synaptic strength. In extracellular field recordings, the plasticity of single neurons is not possible to measure, but the average change in plasticity in the stimulated population is. In order to induce a LTP response, the neurons are stimulated with a few repeated bursts of pulses (10-20Hz) with a short resting period (a few seconds) between them. This fast sequence ensures that the NMDA receptors are activated and leads to a potentiation of the synapse. In extracellular field recordings, several different stimulation frequencies (many of frequencies not naturally occurring) have been used to induce an increase of the synaptic strength.

Typically, when inducing LTP, a great increase in synaptic strength can initially be observed. This increase then gradually drops over the first few minutes of recording. This initial increase is what is referred to as short term potentiation (STP). As the STP effect wears off, the strength in synaptic response stabilizes above the baseline synaptic strength recorded before the stimulation. This lingering effect is the LTP (Figure 13).

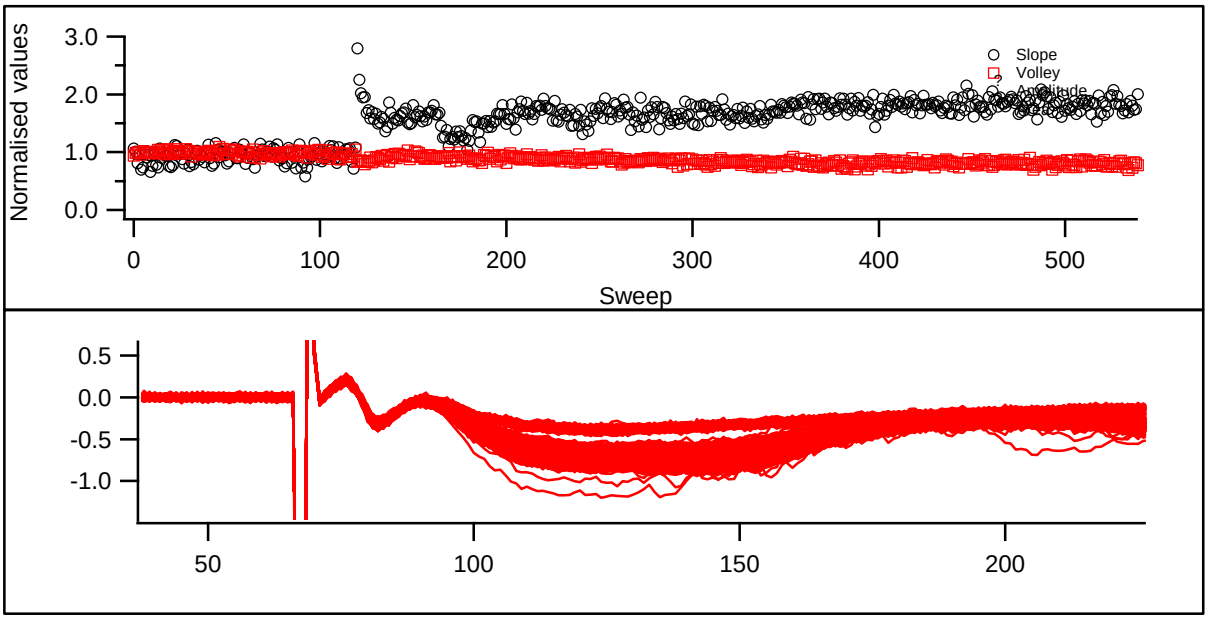

Figure 13: Example of LTP. Top: the slope of the EPSP baseline (black) and the volley (red) plotted for each sweed in the trial. The LTP incucing protocol was run after 120 sweeps. The peak at 121 is the short term potentiation (STP) response in the synapses. It declines rapidly and reaches a stable level later on. That lasting increase is the long term potentiation (LTP). Bottom: The raw data waves measured plotted together. Each line corrsepond to one of the 540 sweeps of the trial.

Age of rats, synaptic silencing and dendritic spines

When performing acute field recordings from hippocampal slices from rat, recordings from younger animals tend to have a much lower magnitude of the recorded EPSP response (relative to the volley) than older animals. Animals of 10 days age usually give an EPSP response magnitude of 1 to 1.5 times the volley, while animals of 20 days age give EPSP responses around 4 to 5 times the volley. This difference in response can be explained by the development over time of AMPA-rich synapses.

It is believed that most glutamatergic synapses are born AMPA-silent. That is, having no AMPA receptors. Coordinated activity with other neurons leads to AMPA activation (the synapses get AMPA receptros incorporated into the cell membrane). In young animals a larger portion of synapses will be silent; and it is hard to get an EPSP response in extracellular field recordings from animals younger than 7 days. When transferring from silent to active i.e. aquiering AMPA-receptors, the synapses pass through an unstable state in which they initially behave as active synapses. Uncordinated activity (like

the baseline sweeps in extracellular field recordings) may cause them to loose their AMPA-receptors and become silent. When no stimulation occurs for a prolonged time the synapses reactivate again.

After an LTP-inducing protocol, many of these synapses are stabilized and will no longer fall silent, resulting in a stable increase in the EPSP response from that moment on. Unsilencing by AMPA-activation of synapses is probably the main mechanism for LTP in young animals.

The silent synapses, like other synapses, are often located on very small protrusions on the dendrites of the neuron. These small protrusions are an immature form of dendritic spines. As the synapse is stabilized, the small protrusion grows in order to accommodate more receptors and evolves into a fully developed dendritic spine.

Method

Below follows a record of all the experimental methodology used in the project. The chapter begins with a brief description and motivation of the hCSF used in all the experiments. Following that the slicing and cultivation are described. After that follows two trials intended to investigate the pH in the incubator and the adding of hepes as a buffer solution to the cultivation medium.

Rats, culture media and slicing procedure

This section describes the animals and the hCSF used in the study and the slicing procedure.

Rats and their age

Culturing of hippocampal slices has been more successful when taking slices from younger animals. Successful is in this case refers to recordings from cultured slices producing results that are similar to recordings from acute slices from rats of the same age. This includes the culture time, e.g. a slice from a rat of 8 days cultured for 4 days should perform similar to an acute slice from a 12 day old rat if synaptic development in the slice has continued in an in-vivo-like manner.

Gähwiler (1981) oserved recordable effects from 8 days old rats, Stoppini et al (1991) used rats of age 2-23 days postnatal obtaining the best results from 8-15 days old individuals, and Caeser and Aertsen (1991) used rats of age 4-7 days. Thus it was initially decided to primarily use Wistar (a laboratory breed of rats commonly used in medical research) rats of age 6-15 in the experiments. However, it would be much more interesting from a future research perspective to be able to conduct studies on slices from older animals, as it opens a wider window of possible investigations.

The success of the first trial culture experiment suggested that it would be possible to obtain results from rats of older age as well and the plan was changed to include some rats in the range of 16-25 days age.

The rats used in the project were bred at the laboratory for experimental biomedicine at the University of Gothenburg. Normally rats are bred such that new litters are born around the same day of the week every week. This presented a problem with working schedules that made random selection by age for the experiments unfeasible. Also, some weeks, there were few new-born rats and one week all new-born died. To be able to still conduct any experiments it was decided to go with whatever age of rats were present in the acceptable age range when starting a new culture, and if possible, try to get close to the same number of rats of ages 6-10 and 11-15.

hCSF

In all experiments conducted in this report the hCSF used was donated from patients with normal pressure hydrocephalus (NPH). Samples were drawn from patients via lumbar puncture by neurologists at the Sahlgrenska University Hospital in Gothenburg, Sweden. The samples were pooled and stored in a freezer at -80 °C. The NPH-hCSF was used because of it's availability. Ideally hCSF from healthy donators should have been used for all experiments. However, that requires extensive donations of hCSF. Since previous studies at the department by Björefeldt et al (2015) found that hCSF from NPH-patients and healthy individuals had the same effect on pyramidal neurons, it was deemed close enough to the ideal to motivate the use of it instead of going through the time-consuming steps of donations.

Slicing

Wistar rats of post-natal age ranging from 7 to 27 days were sedated with isoflurane, decapitated and both brain hemispheres were extracted. The extracted hemispheres were quickly placed in GACSF (2.5 mM KCl, 1.2 mM $CaCl_2$, 7 mM $MgCl_2$, 26 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, 11 mM D-glucose) bubbled with a 5%CO2 95% oxygen gas mixture at 3 °C. The hemispheres were subsequently glued to an ice-cold plate, re submerged in the GACSF at 3 C and cut into 400 µm thick slices using a Vibratome™ microtome machine.

The hippocampi were cut out from the slices using syringe needles and placed in ALM medium (119 mM naCl, 1 mM NaH_2PO_4, 26 mM $NaHCO_3$, 3 mM KCl, 10 mM glucose) bubbled with a 5% CO_2, 95% oxygen gas mixture at room temperature. This was done in order to allow for cell recovery from the trauma of cutting. The resting period ranged from 30-60 minutes for the slices before culturing. Resting times in ALM medium for field recordings from acute slices ranged from 45-240 minutes, with the shorter times applying to the first slices recorded from.

Cultivation

Cultivation procedure followed the general procedure described by Opitz-Araya and Barria (2011). Culturing was done in the wells of 6-well culture plates on two types of membranes. Some early cultures during the practising phase were made on Millipore™ high walled membranes, since the desired membranes were not available for a brief period. The regular cultures were made on Millipore™ low walled membranes (transparent when wet). To prevent growth of bacteria in the cultures, all samples were supplied with penicillin, streptomycin and nystatin.

Protocol

Prior to culturing, samples of the culture media to be used were prepared. Three different media were used. Medium 1 (hCSF): To 1.1 ml NPH hCSF was added 11µl penicillin/streptomycin (5000units penicillin/5mg streptomycin) and 7µl nystatin (1000 units/ml). Medium 2 (hCSF+1mM hepes) was prepared identical to medium 1 with the addition of hepes to a 1mM concentration. Medium 3 (NB) was prepared by mixing 50 ml Neurobasal, 500 µl L-glutamine, 500 µl penicillin/streptomycin, 1 ml B27
and 32 µl nystatin (1000 units/ml). The samples of all three media were aliquoted in appropriate volume (1.1 ml/sample) Eppendorf tubes, vortexed for a few seconds and stored in a freezer at -20 °C. Samples of the appropriate culture medium were thawed to room temperature prior to preparation of cultures. Henceforth in the report, medium 1 will be referred to as hCSF, medium 2 as hCSF+1mM hepes and medium 3 as NB in the report when referring to culture medium.

Early project cultivation

A clean area was prepared on which to conduct the preparation by cleaning it thoroughly with 70% ethanol and letting it dry. Culture wells were marked up and prepared by pouring prepared culturing solutions (hCSF, hCSF+hepes, or NB respectively) in them. A transfer well in which slices were placed on the culturing membranes was filled with 1.1 ml Gibco® HIBERNATE® Media. The membrane was carefully placed in the transferring well to avoid bubbles of air stacking underneath. Slices were transferred from the ALM resting media to the culture well using a glass pipette and excess ALM medium was carefully removed with a pipette. Up to 5 slices were cultured on the same membrane. After removing excess ALM, the membrane was moved to the culture well using a sterile forceps and the next well was prepared in the same manner. The lid was put on the culture plate and it was moved to a Thermo Forma incubator and incubated at 37 °C and 10% CO_2 in the air inflow.

Changes along the course of the project
The hibernate in the transfer well was exchanged for the actual culture media used from experimental ID 2016-03-29-1-p8 and forward.

Nearing the end of the project, a new incubator with better regulation of the air moisture arrived at the lab. In conjunction with this, a special room was prepared for all sterile work with cultures. The protocol for slice preparation changed accordingly and all further work from experimental ID 2016-04-26-1-p16 was conducted in a LAF-bench in the sterile room.

Slices were transported in the ALM media for ~2 min to the room prior to transfer to the wells. During this time no air bubbling thorough the ALM was made.

pH and osmolarity

Below follows the different trials made with respect to pH and osmolarity in this study.

Pre-trial screeningof pH at different CO_2-inflow to incubator
The pH in the CSF under physiological conditions is slightly lower than that in the plasma. It lies in the range of 7.3-7.4 (Andrews et al, 1994). Stoppini et al (1991) and Opitz-Araya and Barria (2011), tried to maintain a pH at around 7.3 under cultivation conditions of 37 °C and 5% CO_2 mixed with 95% air in the inflow to the incubator. Based on this, it was decided that a pH not deviating too far from 7.3 was desirable. Due to problems with stability in pH and osmolarity in the the experiments by Ignell (2015), conducted at the same department, the decision was made to run a screening experiment to measure pH and osmolarity in the incubator to be used. The screening was run without any slices, but otherwise the intention was to keep the conditions identical to real cultures.

Another way of achieving a more stable pH in solutions is to add a buffering substance. In cell and tissue culturing it is common to use hepes, because it buffers at physiological pH levels (range) and is metabolically (relatively) inert. It was decided to add three concentration levels of hepes to the screening trial of pH in the incubator in addition of unmodified hCSF. The concentrations used were a low level of 1mM, as used by Ceriottia et al (2007) when cultivating cells in serum, which contains naturally occurring buffers to begin with, just like hCSF does; a medium level of 5mM; and a high level of 10mM, as it is common to use concentration ranges from 10 to 25 mM when culturing neurons in non-serum media.

A 6-well culture plate was prepared at room temperature with 1,1 ml solution in four wells according to the following scheme: Well 1: hCSF, well 2: hCSF + hepes 1mM, well 3: hCSF + hepes 5mM, well 4: hCSF + hepes 10mM.The plate was placed without a lid in the incubator at 37 °C with 5% CO_2 in the air inflow and incubated for 28h. Readings of pH were made at 7 occasions during the incubation. After the fifth reading at 25h incubation the inflow was changed to 10% CO_2 for the last two readings. An initial osmolarity reading was made on the pooled hCSF used for preparation of the solutions in the trial and a final omsolarity reading was made after the 28h incubation for all wells containing hCSF.

When the real cultivation began, it was discovered that cultures would normally be incubated using a lid on the 6-well plate. The uncertainty of the screening trial (see **Error! Reference source not found.**) still left the question of whether hepes would enhance the culturing by stabilizing pH at a desired level unanswered.

Hepes as additional buffer
It was decided to do a follow up on whether adding hepes contributed significantly to the pH buffering of hCSF or not. To investigate this in a more systematic way than the initial screening, four culture experiments were set up with half of the wells using hCSF and half of the wells hCSF 1mM hepes.

Data was collected from all cultures made during a 3-week period and subsequently analysed by ANOVA. The sample size after 3 weeks was small and was deemed insufficient to draw any conclusions. It was desirable to know as early as possible if hepes could be excluded from the culture medium and the actual cultures rendered few pH recordings, as a recording could only be made after the recording of the last slice. Thus it was deemed appropriate to complement the actual culture data with some more measurements on a "dummy" culture containing no slices. Had more time been available for the project, it would have been better to get more data from proper cultures.

To generate more pH recordings, two extra plates with dummy cultures were prepared identically to real cultures, 2 wells hCSF +2wells hCSF+1mM hepes/plate, but without any slices, were prepared and incubated at 10% $CO_{2\ for}$ 121 hours. pH recordings were made at three occasions for plate 1 and 2 times for plate 2.

Osmolarity and osmolarity change due to evaporation in hCSF
The osmolarity change due to evaporation in earlier experiments had been found to vary considerably. Thus it was of interest to monitor the final osmolarity in the cultures incubated. Osmolarity was recorded after the field recordings of every slice in a well in the experiments. This resulted in different recording times for the different cultures during the project. Initially only the final osmolarity was recorded. In the later staged of the project, the starting osmolarity of cultures was recorded as well by using excessive culture medium and pooling off the overshoot of the 1.1 ml used in the culture well for osmolarity and pH recording. The osmolarity data was analysed using a simple plot of osmolarity over time.

Extracellular field recordings
Field recordings were made at a recording station at the department of Neurophysiology at the University of Gothenburg. After placing the slice in the recording well, two tungsten stimulation electrodes and a recording electrode were placed in the surface of the slice. The recording electrode consisted of a silver coated platina electrode submerged in a 1M sodium chloride solution inside a fine tipped glass pipette. Pipette resistance aimed for was 3-5MOhm, but recordings were found to still work outside of this range, so pipettes in the range of 1.5-7 MOhm was deemed acceptable during actual recording. During recording, the slice was submerged in a recirculated perfusion bath of 100 ml new Ringer (124 mM NaCl, 3 mM KCl, 26 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 2 mM $CaCl_2$, 2mM $MgCl_2$, 10 mM glucose) with 50 µl 200 mM PTX (picrotoxin, a neurotoxin blocking $GABA_A$ receptors) added.

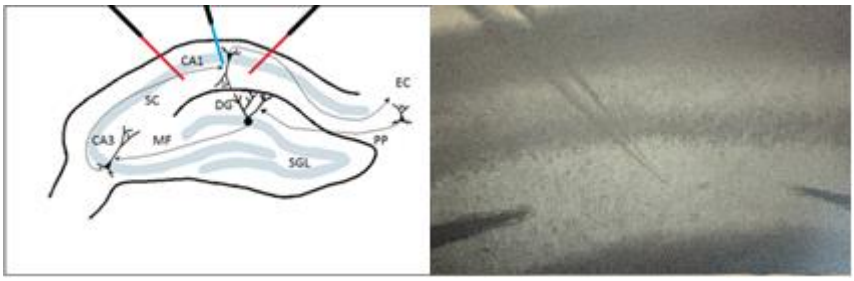

Figure 14: Left: Schematic picture over electrode placement. Right: Actual electrode placement in an acute slice.

A baseline of 240 sweeps total, 120 from each stimulation electrode, at 0.2 Hz was made for 10 min. After the baseline, a LTP inducing protocol consisting of 5 repeats of 20 pulses at 100 Hz with 8s between the repeats was run. After the LTP induction, a recording of the response at 0.2 Hz was made for 35 min. Stimulation strength started in the range of 20 μV and was raised or lowered according to the response. In some cultured slices, stimulation strength of up to 320 mV was used in order to get response above noise level.

DiI dying of slices

It was decided to do a test the DiI-method utilized by Caeser and Aertsen (1991) in a study where several dyeing methods were used in conjunction in order to investigate hippocampal slice morphology. The dye fluoresces red (~580 nm) when under green light (~550 nm). The hope was to investigate if the axon structure of the *Schaffer collaterals*was intact in cultured slices or if neuronal "rewiring" occurred. If extensive rewiring occurs at least in some cases, it could explain strange field recordings sometimes obtained.

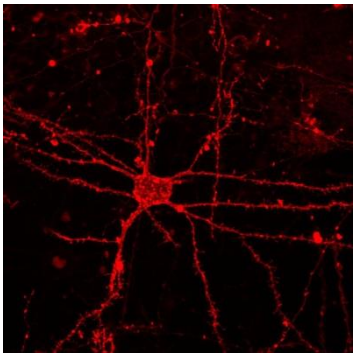

Figure 15.Example of DiI staining of a neuron. This example is from the cortex in a mouse. The soma and the dendrites are distinctly red against the non-fluorescing background.Image: Tariq Chohan (2012).

Samples for DiI imaging were prepared using two different protocols. Protocol 1: Slices were washed in PBS buffer 3 times. After washing, the slices were fixed in histofix for 1h. DiI crystals were placed at the normal recording site of the slice and left to rest in the dark for 5 min. The slice was then washed three times in PBS+$PbSO_4$ and stored dark for at least two weeks. Protocol 2: Slices were washed in PBS buffer three times. DiI crystals were placed at the normal recording site of the slice and left to rest dark for 5 min. Slices were then washed three times in PBS. After washing, the slices were fixed in histofix for 1h. After fixation, the slices were washed three times in PBS+$PbSO_4$ and stored dark for at least 2 weeks. After storing dark, the slices were examined under a light microscope.

Recording and analysis of data

It was deemed that an ANOVA test was appropriate for the hepes experiment as the variation of the mean was of interest and it was unknown if there would be a change up or down in the mean by adding hepes. The data from the extra dummy-trial was added to that of the four real cultures testing hepes addition and analysed with an ANOVA test in Microsoft excel.

Field recordings were analysed in several steps. First, they were visually analysed on the spot during recording and if no volley was detected even after cranking up the stimulation strength to the maximum the equipment could produce, no recording was made for the slice.For the fields recorded, the baseline and the response were concatenated into one file and separated in left and right electrode channels for analysis in IGOR-pro. In IGOR-pro the sweeps were examined more thoroughly and volley and EPSP slope (time-derivative of the signal for the short intervals of volley and EPSP respectively) was calculated.

The trials were subsequently classified as either successful (giving an EPSP response) or failed (not giving any detectable EPSP response) and differences between treatments was examined using fisher's exact test and/or a chi-square test.

Results

Below the results from the study are presented under the sections pH and osmolarity, observations during recordings and recorded results.

pH and osmolarity

Pre-trial screeningof pH at different CO_2-inflow to incubator

The results from the screening trial of incubator pH and osmolarity are presented in Table 1 and Figure 16 below. As stated in the method chapter, the screening trial was conducted without a lid on the culture plate. This affected the final osmolarity, as evaporation of the culture medium is much higher without a lid on the culture plate. An ANOVA analysis was performed for the difference of the means for the 5% CO_2-level. No significance was found (p-value: 0.396675).

time (h)	%CO2	hCSF	hepes 1mM	hepes 5mM	hepes 10mM
4	5	8,50	8,12	8,13	8,23
6	5	7,97	7,72	7,67	7,79
21	5	7,59	7,78	7,72	7,46
24	5	7,93	7,88	7,57	7,43
28	5	7,97	7,73	7,57	7,74
30	10	7,53	7,34	7,30	7,31
32	10	7,73	7,70	7,60	7,62
mean 5% CO2		7,99	7,85	7,73	7,73
mean 10% CO2		7,63	7,52	7,45	7,47
start osmol		297,00			
final osmol		689,00	711,00	661,00	666,00

Table 1: Results from the initial screening experiment. The CO_2 percentage in the incubator is listed along with the pH reading for each sample. Times for recordings are rounded to nearest full hour. The mean pH for each sample has been calculated at both CO_2 levels. As can be seen, it lies higher at 5% CO_2 than at 10%.

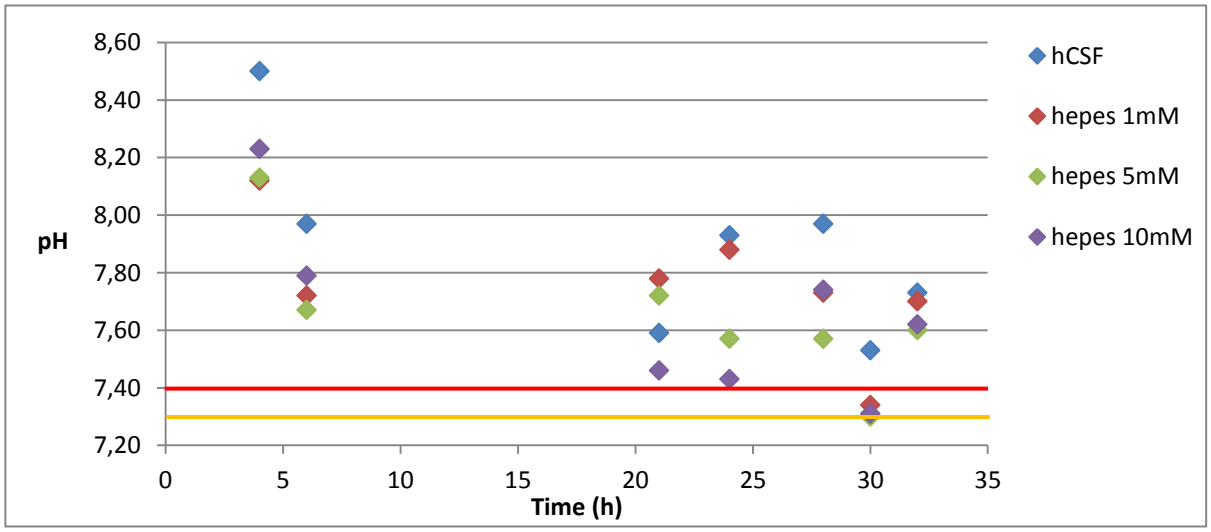

Figure 16: Plot of pH for each sample in Table 1 as a function of time. Initial pH values were rather high and stabilised towards the end of the recording. The orange line indicates the desired pH level of 7.3 and the red line the upper acceptable limit at a pH of 7.4

Hepes as additional buffer

The results from the test of whether addition of 1mM hepes contributes to the buffering when added to hCSF samples for culturing. The initial screening trial indicated that a difference could be present but sample size to low to detect it so a follow up trial was made during culturing. Initial follow up trials containing samples with both hCSF and hCSF with 1mM hepes added are presented in the first section of Table 8. The second section contains additional trials using only hCSF as culturing medium and the third section contains data from a trial run with no cultured slices in it. The trials in the third section were made in order to obtain more data quickly as the culture time was a limiting factor for sample sizes and it data was needed to make a decision on whether to keep adding hepes or not. Data from all three sections of the table are presented graphically in Figure 17.

An ANOVA analysis was performed using all data from Table 8 in the appendix. There was no evidence for a significant difference in mean pH (hCSF mean pH 7,43, hCSF+hepes 1mM mean pH 7,51, P-value 0,107445).

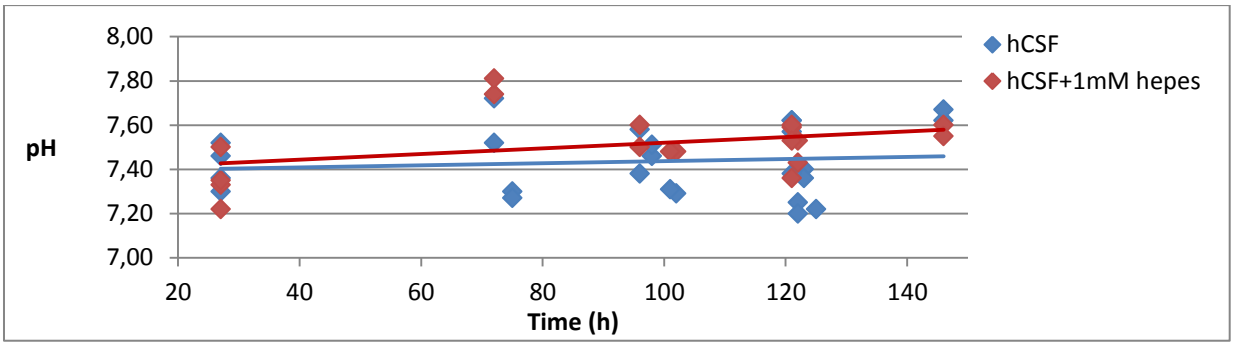

Figure 17: pH plotted as a function of time for all the data points in Table 8. Trendlines calculated with linear regression for respective dataset is represented by solid lines. Linear regression: R^2 (hCSF)= 0,0128, R^2(hCSF+hepes) = 0,1366.

Osmolarity

Results from the osmolarity measurements from all trials are presented as a graph below. In this region the change in osmolarity over time is approximately linear. The rate of change over time is not so high that it gave cause to worry about the osmolarity, but the variation between samples is higher than desired. No further analysis of the osmolarity was made.

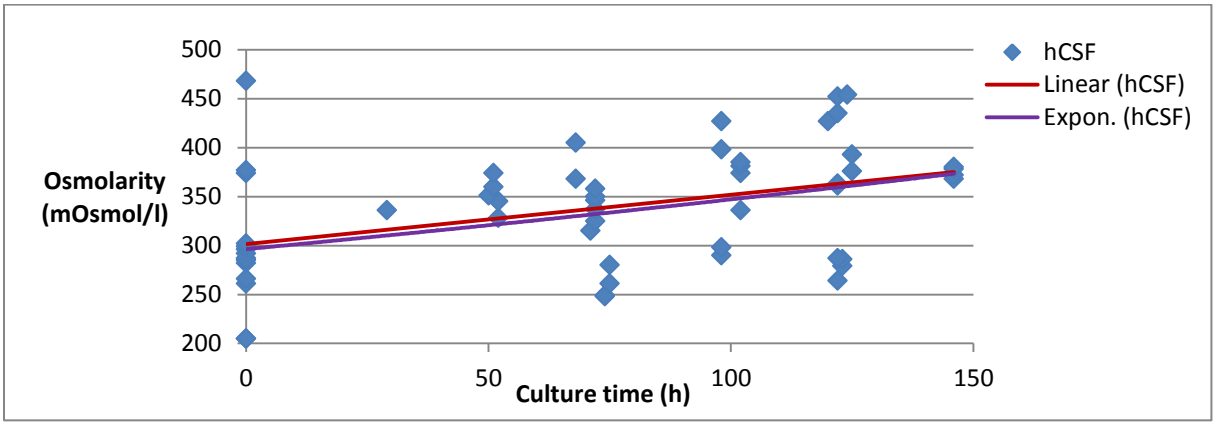

Figure 18. Osmolarity plotted as a function of time in culture. Included in the graph are all hCSF and hCSF+1mM hepes data points. NB pointswere excluded. Linear (red, R^2 = 0,1672) and exponential (purple, R^2 = 0,1751) regression lines has been added to the graph showing that at this low osmolarity, the difference is small enough to assume that osmolarity change over time is linear. Further, it can be seen that the span of data points is largest at the starting measurements, indicating either that initial measurements were more inaccurate or that there is some cause for stabilization of osmolarity after some time in culture.

Culturing and recording observations

The culturing process proved to be difficult. A large portion (60/177 ≈ 34%) of cultured slices never made it far enough in the process to get any extracellular field recording data from them. Some of the scrapped slices simply were too fragile, while other slices were lost because they had been placed too close to the membrane edge for the membrane to be cut without damaging the slice. Placement of slices was crude using the transfer pipette and some transfers resulted in bad slice placements that were only partially remedied by moving slices on the membranes. A few slices were dropped from the glass pipette used for transfer of slices and two whole batches were lost when we had problems with the glass pipette puller for recording pipettes for a few days, making field recordings impossible for that period. These scrapped slices are included in the total spreadsheet in <appendix> but are excluded from further results presented below. The first part covers some photos taken during the project and explains some of the culturing process work. Following that are some tables and graphs comparing different factors.

Recordings were not as straightforward as they are in acute slices. One problem was the placement of the electrodes. In acute slices, the layered structure of the hippocampus is easy to see as distinct bands of darker and lighter colour. This pattern grew more obscure over time as the slices were cultured and it grew increasingly harder to find good stimulation and recording sites. Another problem was that some slices needed significantly higher stimulation than is normal in acute slices to give recordable responses. In acute slices it is common to use stimulating currents from 10 to 30 µA, while some cultured slices needed currents in the range from 150 up to 320 µA.

Figure 19. Left: overview of the slicing process. Two hemispheres have been glued to the plate and are being cut in 400 µm thin slices. Right: Zoomed in portion of the left picture showing the location of the hippocampus (red ellipses). Looking carefully, the hippocampus cross section outline is barely visible. The hippocampus was subsequently cut out from the slices using syringe needles.

Figure 20. Left: Layout of a typical culture plate during cultivation. Typically two or four wells were used for cultures. Right: Zoomed in image from another culture plate. The hippocampal slices have been placed on the membranes. Wells are filled with culture medium below the membrane and the bottom side of the membrane is in contact with the medium. Capillary forces bring the culture medium up to the slices through the permeable membrane surface.

Figure 21. Example pictures of electrode placement in cultured slices (left and middle) and an acute slice to the right. The thick black points are the stimulation electrodes and the transparent one is the recording glass pipette containing the silver electrode submerged in a solution of 1M sodium chloride. In the acute slice to the right, the cinnamon rol –like banded structure of the hippocampus is clearly visible. This makes placing the electrodes where they stimulate the Schaffer collateral axons from CA3 to CA1 in the hippocampus easy. The bands of the cultured slices were less obvious. This degradation was present to various degrees in many of the cultured slices, with the more extreme cases being uniformly grey, making electrode placement much more difficult.

Extracellular field recordings

When recording extracellular fields from the cultured slices, the results varied. Some slices did not respond at all when stimulated. All that was observed was the stimulation artefact. When a slice did not respond with a clearly detectable volley at simulation currents up to 30 μA, the stimulation was gradually cranked up to the maximum of the equipment (30 mA). This stimulation strength makes the extracellular fluid at the electrode boil locally, and severely damages the cells located close by. These extreme stimulation levels were only used at slices that failed to respond at lower stimulation. Slices that did not produce a volley at 320 μA did not respond at higher stimulation either. These slices were considered "dead" and no recordings were made from them. Other slices did produce a fiber volley, but failed to produce any other detectable signals during stimulation. These fields were not recorded either, but the volley response was noted.

The highest stimulation strength used in any recording was 320 μA, which is a tenfold increase compared to normal recordings in acute slices. It was still used in some cases, as it was suspected that different resistances in the cultured slices compared to acute slices could account for a lack of detectable responses at lower stimulation. No fields recorded at 320 μA showed the typical signs of overstimulation that this stimulation magnitude causes in acute slices, and it was taken as an indication that the higher stimulation was appropriate.

While many slices gave no response at all, a few slices did respond as expected from acute slices with a detectable EPSP field and some even with an LTP response after LTP-inducing stimulation. These fields looked almost like normal fields from acute slices in some cases. Other slices did respond with weird looking fields that are normally not observed in acute slices. Some examples of response fields are presented below.

Evaluation of the responses was made both visually and by computational analysis using IGOR-pro and Microsoft Excel. The time derivative of the linear part of the descent of the response curve was calculated for the volley and the possible EPSP response for each sweep recorded. A field recording from a slice was considered to have an EPSP response if the detected ratio of the response and volley was at least 0.1 corresponding to a response magnitude of 10% of the volley and the field also did look fairly close to what is expected from an acute slice.

Very few of the slices did show an LTP following LTP-inducing stimulation. This was also evaluated both visually and computationally. A response was classified as an LTP if the ratio of [baseline EPSP]/[EPSP at 30 min after LTP induction] exceeded 1.2 and the field response was not too weird looking. This corresponds to an average of 20% increase in synaptic strength.

Beginner's luck experiment

The first culture ever made during the practise phase of the project, before starting serious trials, resulted in the best recording (as in having the field most similar to an acute recording) during the whole project. For the field recording of this trial no PTX was used, resulting in a longer phase of positive polarisation of the membrane potential. This experiment is extraordinary also with respect to rat age, as most successful slice cultures are from rats between 7 and 10 days old and it gets increasingly difficult to get slices to survive in vitro as the rats grow older (Rats and their age).

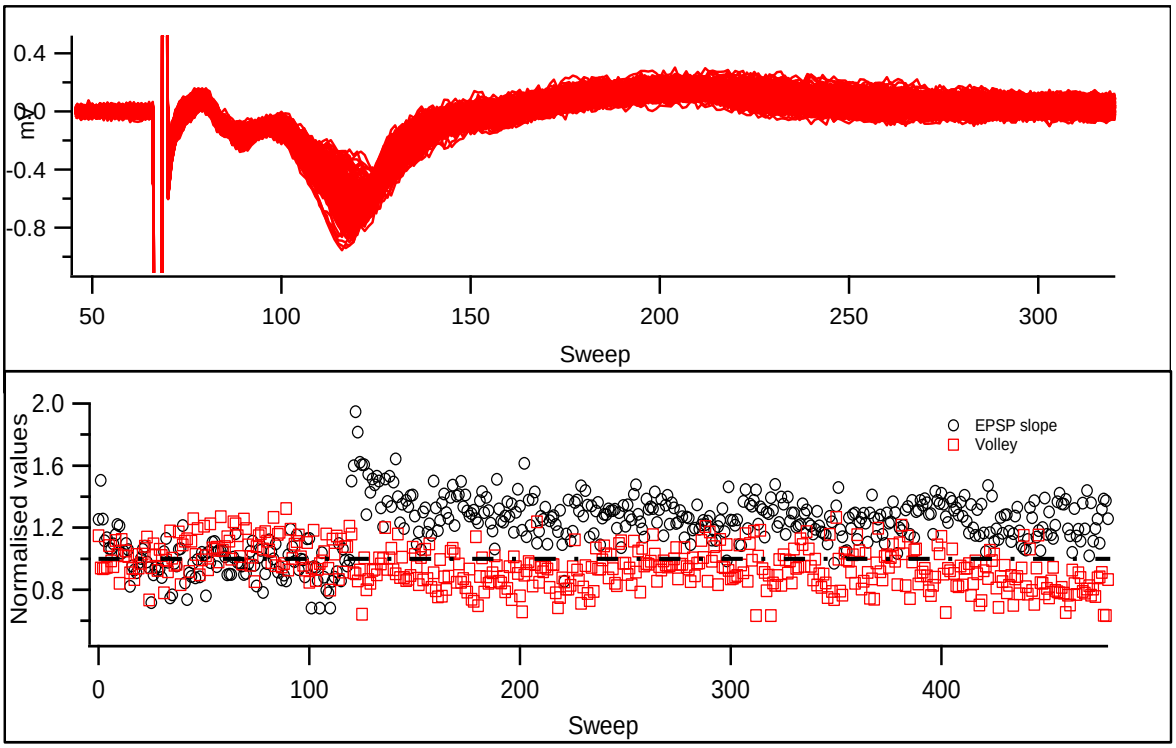

Figure 22. The most successful trial was made during the practise phase. A slice form a 27 day old rat was cultivated for 4 days. Top: All sweeps from the trial in one graph. The volley is clearly detectable as and the variation in magnitude is low. The EPSP response is more diverse, corresponding to STP and LTP increases in magnitude compared to the baseline. The long duration, positive potential recorded after the EPSP peak is typical for trials where PTX is not added.Bottom: Normalised EPSP slope and normalised volley slope plotted together. The typical pattern of LTP induction can be seen with the baseline at a steady level up to sweep 120. After that there is a sharp increase corresponding to the STP, which gradually declines until the steady long term increase of the LTP remains.

Weird fields

Several weird looking fields were obtained during the extracellular field recording. Quite a few of the fields from slices cultured in NB showed these properties. Slices grown in NB also tended to respond to lower stimulation strengths. Below follows a few examples of weird fields from different recordings. They are good representatives for the type of odd fields recorded, but do not cover the full range of observations

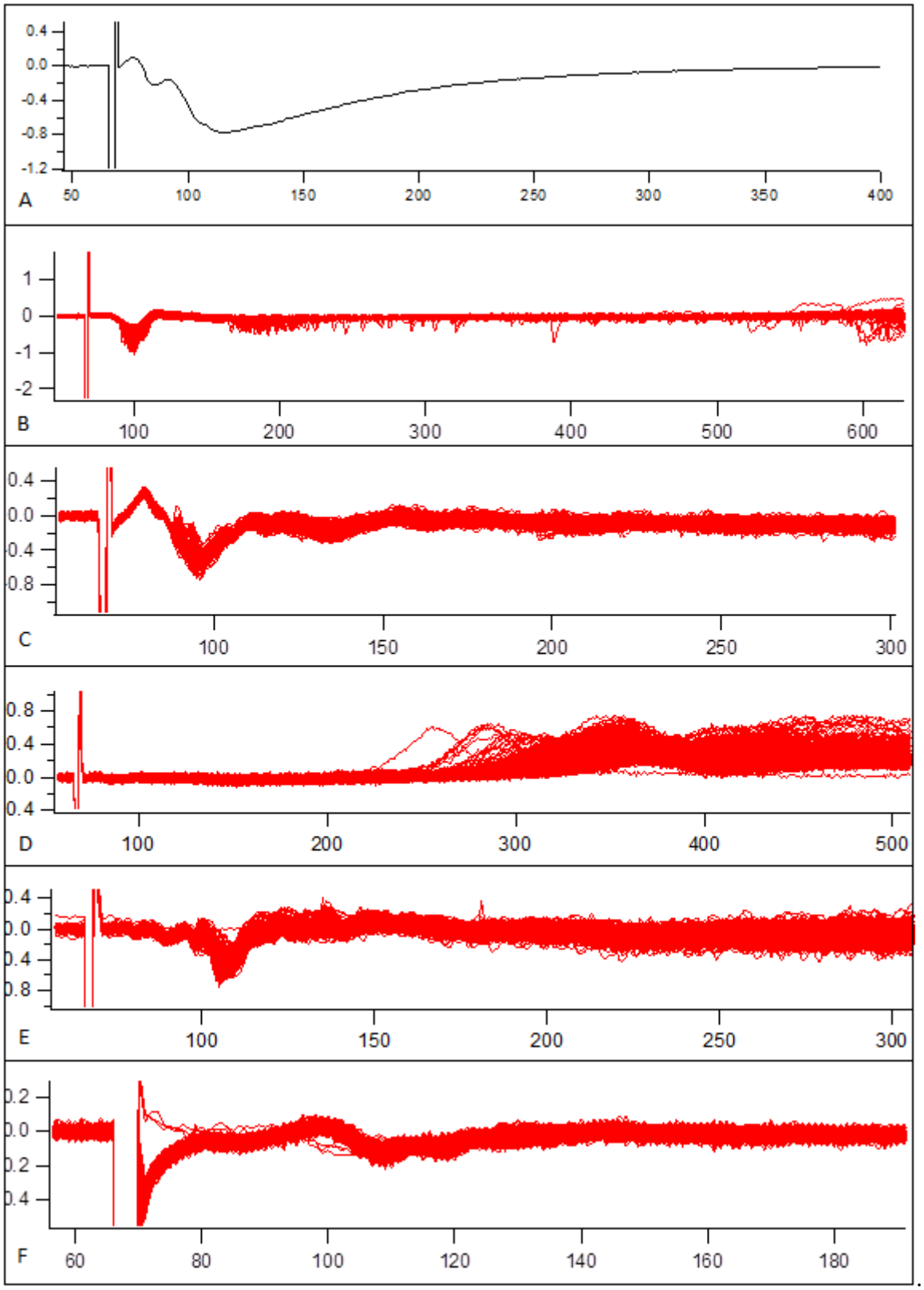

Figure 23. Five examples of odd fields and an example sweep for comparision. The recorded fields are zoomed to different scales to better show what is wrong with them. ***A****:Example sweep from a good field recording. The volley and the EPSP are distinct and easy to detect.* ***B****: Field recordings from a slice from a rat of age 8, 3 days in NB culture. The field has no clear volley.****C****: Field recordings from a slice from a rat of age 11, 3 days in NB culture. Rhe field has no disctinct volley and the EPSP waves do not overlap well.****D****: Field recordings from a slice from a rat of age 12, 2 days in NB culture. The volley and EPSP are minimal, while a late response is gigantic relative to them.* ***E****: Field recordings from a slice from a rat of age 11,4 days in hCSF. Sweeps do not overlap well, and individual sweeps look like they have double volley and double EPSP.* ***F****: Field recordings from a slice from a rat of age 8, 2 days in hCSF. No distinct volley and a non-normal response.*

Outcomes of the field recordings

Using the criteria described above for LTP and EPSP, the successful trials were counted for each level of animals, culturing wells and slices. Excluding scrapped slices from the calculations, animals = 29, culture wells = 49 and slices = 117. Considering only LTP responses as successful, the total number of successful slices was 4, the number of individual wells these came from was 4 and the number of unique animals was 4. This gives the success rate at the LTP cut-off level for successful animals as 4/29 (14%), for successful wells 4/49 (8%), and for successful slices 4/117 (3%). If the success cut-off is instead set to EPSP response, the rate for animals is 14/29 (48%), for wells 17/49 (35%) and for slices 30/117 (26%).

	total	LTP	EPSP
animals (l)	29	4	14
success rate		13,79%	48,28%
wells (m)	49	4	17
success rate		8,16%	34,69%
slices (n)	117	4	30
success rate		3,42%	25,64%

Table 2: The total number of trials at animal level, culture well level and individual slice leveland the corresponding number of successful trials.

Due to the low rate of successful trials, it was concluded that an extensive analysis quantitative analysis in order to compare response magnitudes was not feasible. It was still deemed of interest to look into some factors to see if any conclusions could be drawn. The main question of interest for this study to answer was if the method utilized with hCSF as culture medium is viable to culture slices in. The outcome using hCSF as culture medium was compared to cultures using NB. Another parameter of interest was if the change of transfer medium from hibernate to the culture medium during the project had any measureable effect. A third parameter of interest to analyse was the age of the rats

Analysis of culture medium

Since the addition if hepes did not have any significant effect, the cultures with hCSF and those with hCSF+1mM hepes were pooled for further analysis regarding the culture medium and compared to the slices cultured in NB. Outcomes for the EPSP cut-off for a success were analysed at the well level. Since the sample size was small, it was deemed appropriate to use Fisher's exact test to evaluate whether there was any difference between the samples or not. A chi-square comparision was also performed. No significant difference between hCSF and NB medium could be detected at the 95% confidence level.

	EPSP	total	Success rate
hCSF	12	39	0,307692
NB	5	10	0,5
Two Tailed p-value			0.2592749
The Fisher exact test			0.507828

Table 3. Number of successes and total sample sizes at the culture well level comparing hCSF and NB as a culture medium. Bottom row lists the outcomes of the statistical tests performed.

Analysis of transfer well medium

Since the sample size was small, it was deemed appropriate to use Fischers exact test to evaluate whether there was any difference between the samples or not. A chi-square comparision was also performed. Neither test was significant at the 95% confidence level.Bothe the chi-square and Ficsher's exact test were used to evaluate the sample differences. No significant difference could be found. Outcomes were analysed at the well level.

	EPSP	total
hCSF	7	21
hibernate	5	18
Two tailed Chi-square p-value		0.7114514
Fisher's exact test value		1

Table 4. Number of successes and total sample sizes at the culture well level comparing transfer well medium. Bottom row lists the outcomes of the statistical tests performed.

Analysis by age

For analysis of the effect of age on the success rate of cultivation the data was binned into age groups. The majority of trials were made in age groups 6-10 and 11-15. The data in Table 5 shows the breakdown of the results on individual rat, culturing well and single slice level. The culture well level was analysed further by a chi-square test that failed to show significance (p-value: 0.251082) and by visualizing the bin with a bar diagram (Figure 24).

rats		
age group	Total	EPSP
6-10	11	7
11-15	10	5
16-20	2	0
21-25	5	1
26-30	1	1
total	29	14

wells		
age group	Total	EPSP
6-10	19	11
11-15	17	4
16-20	4	0
21-25	8	1
26-30	1	1
total	49	17

slices		
age group	Total	EPSP
6-10	40	18
11-15	44	10
16-20	11	0
21-25	21	1
26-30	1	1
total	117	30

Table 5: Results broken down by age group. Only evaluated at the EPSP level of success.

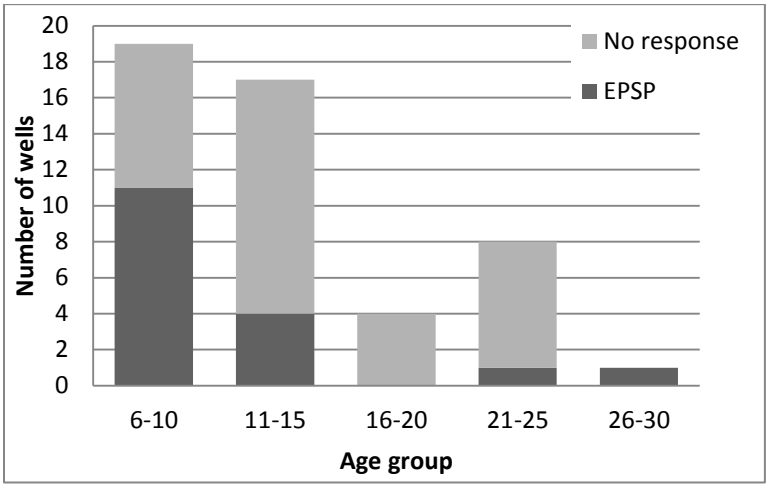

Figure 24 Trials with EPSP response compared to number of trials with no response displayed per binned age group. Total in each group is represented by the bar height. There is a tendency towards more successful trials using younger animals.

Analysis of wells with successful slices more than one day

The original plan included a hope to look for culture longevity and record fields from slices from the same culture well at different days. Due to the low success rate, this hope was never realised and no systematic trials were made. However, by chance, some days there was no time to record from all the slices in a culture and they were left in the incubator. This resulted in fields from some slices from the same well getting recorded at different days. The data set contains recordings from more than one day from 20 of the 56 wells used in the project. Out of these, 4 wells showed successful slices at the EPSP level. This was taken as an indication of a possibility that if slices made it past the start phase of the culture successfully, they could survive for a prolonged time and worthy of further investigation beyond the scope of this project. No statistical analysis was performed on this small data set.

TrialID	age (days)	transfer well medium	culture medium	days in culture	LTP	EPSP
2016-03-18-1-p10	10	hibernate	hCSF	3	no	yes
2016-03-18-1-p10	10	hibernate	hCSF	4	no	yes
2016-03-29-1-p8	8	hCSF	hCSF	2	no	yes
2016-03-29-1-p8	8	hCSF	hCSF	3	no	yes
2016-04-05-1-p8x2	8	hCSF	hCSF	2	no	yes
2016-04-05-1-p8x2	8	hCSF	hCSF	4	no	yes
2016-04-05-1-p8x2	8	hCSF	hCSF	3	yes	yes
2016-04-05-1-p8x2	8	hCSF	hCSF	4	no	yes

Table 6. Table of the trials that resulted in at least EPSP response in a slice on more than 1 day. It occured in 4 cases (of a total of 20 where slices from the same well were examined at different days). The trial with ID 2016-04-05-1-p8x2 used two rats of 8 days age. Slices from each of the rats were prepared by different members of the department.

What about the acute slices?

During the project, recordings were made from acute slices from a few of the rats used to start the cultures. This was made with the intention of having data from acute slices from the same animals to compare the outcome of culturing with. Since the culturing did not result in enough successful LTP trials, the data was never used in this manner. It still turned out to be useful as a benchmark if the author was still capable of performing decent recordings. This was useful to ensure no introduction of excessive experimenter error at the cultured slice evaluation recordings. Of the 14 slices recorded, 13 (=93%) gave an LTP response above the 1.2 times the baseline EPSP used as a cut-off for the cultured slices. Below follow some brief analysis and graphics of some of the acute slice data.

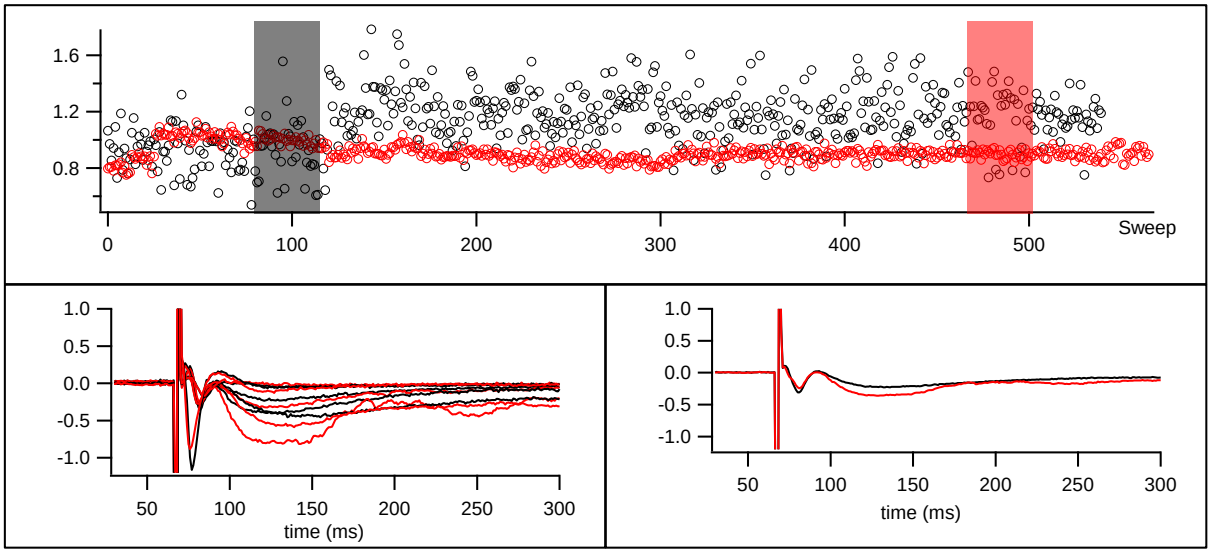

Figure 25. Example of recorded fields from 5 trials with rats of age 11-15 days.Top: The graph shows the average relative EPSP and volley magnitude for each consecutive sweep during a trial of 10 min baseline of 120 sweeps just stimulating with a single pulse at 0.2 Hz. After that the slice was given a LTP inducing burst sequence of 5 repeats of 20 pulses at 100 Hz with 8 seconds rest between the repeats. The sweeps from 121 to 540 were then recorded showing at the same sampling rate as the baseline. The black rings correspond to the EPSP magnitude and the red ones to the volley magnitude. There is a sharp increase in magnitude of the EPSP right after sweep 120, which corresponds to a strong short term potentiation (STP). This strong potentiation quickly declines but at sweep 480 there is still left a substantiallingering effect, the LTP. The black and red shaded areas correspond to the black and red lines in the graphs below. Lower left: 40 sweeps averaged from the sweeps indicated above (81-120 and 481-485 black and red respectively) for each of the 5 rats. Lower right: Average values of all the rats in the left picture. As can be seen, there is an increase in EPSP clearly visible while the volley remains at the same magnitude.

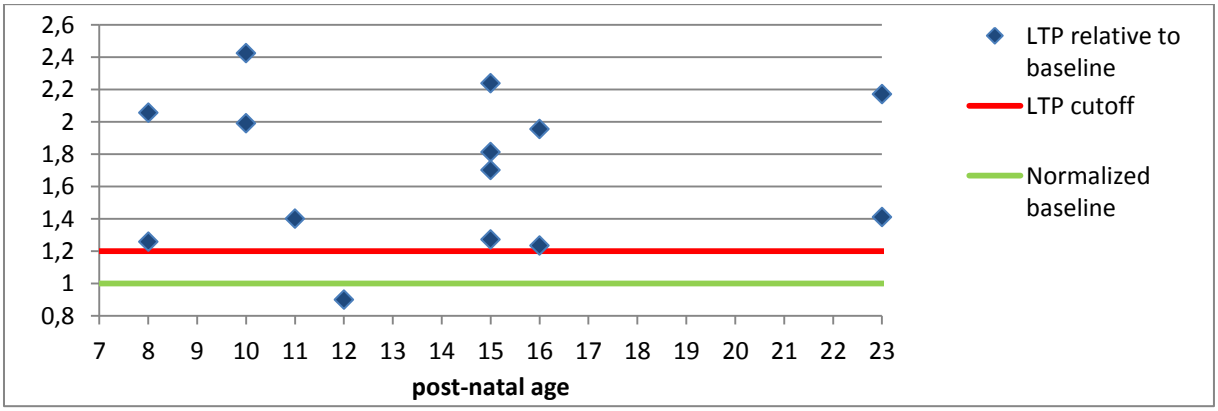

Figure 26. Plot of LTP magnitude measured in acute slices relative to baseline. The green line is the normalized baseline at 1 and the red line is the cut-off of 1.2 used to determine if cultured slices did show an LTP response. As can be seen, only 1 acute slice out of the 14 in the data set did not show any LTP. However, the magnitude of response of the slices from older animals (15,16, and especially 23 days) is rather lo, indicating somewhat lower quality of that particular recording either in slice preparation or in electrode positioning.

DiI dying of slices

In order to investigate whether the axons in CA3 still connected to the CA1 neurons, and that no "rewiring" of neuronal contacts had occurred in cultured slices, a few slices were dyed with the fluorescent dye DiI. A few initial samples were made and analysed under a light microscope after two weeks. The microscope investigation showed some staining of cell membranes, but it turned out that the signal from the membrane drowned in the signal from the crystal fragments remaining on the slice, making it impossible to see the location and extension of the axons.

Due to the long time needed to obtain DiI results and the initial difficulties, it was decided to drop the DiI branch from further investigation during the project. The imaging by DiI remains a future project, as it would be of high interest to visualise if and possibly how the neuronal network in cultured and acute slices differ.

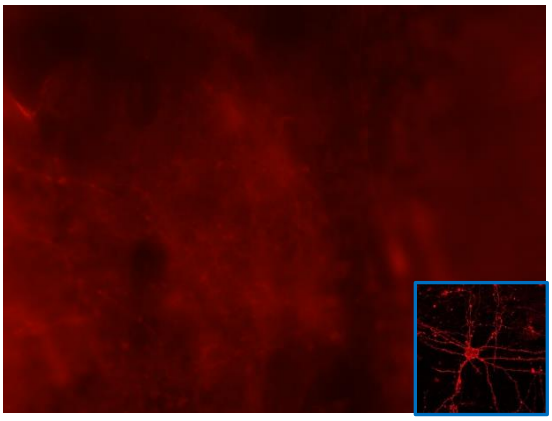

Figure 27. DiI image taken after 2 weeks. As can be seen in the image, some cell membranes including dendrites (the prolonged small lines) are possible to see, but there are no axons clearly visible. What can also be seen is clear hot-spts (bright red) where remnants of the crystals used to dye were still left on the slice. The intensity of these spots was so high that even if there were more subtle signals from dye in axons it was impossible to detect it above that noise. Lower right corner: Picture (same as shown in higher resolution in Figure 15 in the right corner) for comparison.

Discussion

Below follows a discussion of all the results followed by some suggestions on how to follow up the results. Last follows a more general discussion regarding the work on this project and the relation to learning, teaching and didactics.

Pre-trial screeningof pH

The initial "quick and dirty" screening did not produce any detectable significance in the mean pH of the recordings. However, the graphical analysis and the table suggest a trend towards higher pH in the hCSF sample than in the hepes buffered ones. This was taken as indication that more data was needed, but there was also an urgent need to get started on the cultivation experiment early, so it was decided that testing the effect of adding 1mM hepes to half of the upcoming cultures was a reasonable follow up. The higher concentrations were discussed and discarded, as no studies published using hepes addition of that magnitude to naturally buffering media (serum, plasma etc.) was found.

The pH seemed stable at a slightly too high averages (in the range of 7.73-7.99 for the different culture media), indicating that the partial pressure of the 5% CO_2 in the air inflow was a bit too low to obtain physiological conditions with a pH at around 7.3 (Andrews et al, 1994). Increasing the inflow of CO_2 to 10% brought the mean pH down to the range of 7.45-7.63 for the different media. While this was still not in the 7.3 range it was close enough, as a higher inflow of CO_2 to the incubator would make use of the incubator impossible for other experiments. Even the 10% inflow was limiting the use of the incubator for other experiments during the culturing period.The additional CO_2 also has the added side effect of lowering the oxygen concentration, and it was unknown whether the 10% was already causing too much of a disturbance.

Hepes as additional buffer

The results from the follow up experiment on the effect of adding 1 mM hepes arrived at a mean difference between the hCSF pH and the hCSF+1 mM hepes cultures of 0.08 units (close to the measuring accuracy of the pH meter, given that it is correctly calibrated) and the ANOVA failed to detect any significance. Based on this, it was concluded that even if there could be a difference between hCSF and hCSF with 1mM hepes added, that difference is so small that it will make no practical difference during cultivation. Thus it was decided to drop hepes from further experiments. After the conclusion, no more cultures were started using additional hepes in the hCSF and in analysis of the data the hCSF- and hCSF+1 mM hepes- cultures were pooled together.

Osmolarity

Lack of humidity saturation in the incubator air could cause extensive evaporation raising the osmolarity too fast in culture media. An investigation of osmolarity was initially made to keep track of whether the incubator humidity was stable or not, as the study by Ignell (*2015*) had revealed that several cultures had really high values in osmolarity (over 600 mOsmol/kg, compared to physiological conditions of around 300) after a few days in culture. The results of the monitoring of the osmolarity reveal no extreme changes over time. The rate of change is approximately linear in the concentration region of interest and no observations as extreme as 600 mOsmol/kg were observed even after 5 days in culture. This was taken as an indication that the change in osmolarity in the cultures was not a huge problem.

However, there is a considerable variation in osmolarity between the samples and the starting osmolarity of cultures did vary more than late culture measurements. Opitz-Araya and Andres Barria (2011) states that it is imperative to keep starting osmolarity in the range of 290-310 mOsmol/kg, in order to produce successful cultures using artificial culture media. Regulating the hCSF osmolarity

was not considered during the project, but could be a future scope. Still, it could be more difficult than regulating the osmolarity of an artificial medium, as the individual components (and their concentrations) are unknown and should the osmolarity be too low it is difficult to adjust it upwards. Still, it is desirable, to be as close to 300 mOsmol/kg in starting osmolarity as possible.

Another thing to take into account is that the variation in starting values is much higher than later measurements from cultures. One explanation could be that the culture stabilized the value after a few days, which (according to the author's guess) is highly unlikely, since consumable parts of the medium such as glucose only contribute to a fraction of the total osmolarity of hCSF. Another (far more likely in the eyes of the author) explanation is that the initial pH readings were less reliable. Possibly due to inaccurate sample volumes, as bubbly fluids are hard to measure with accuracy using a pipette and pipetting the culture media into the culture wells sometimes caused bubbles.

Stimulation strength

The highest stimulation strength used in any recording was 320 μA, which is a tenfold increase compared to normal recordings in acute slices. It was still used in some cases, as it was suspected that different resistances in the cultured slices compared to acute slices could account for a lack of detectable responses at lower stimulation. No fields recorded at 320 μA showed typical signs of overstimulation, with extensive firing of the neurons during the EPSP that this stimulation magnitude causes in acute slices. This was taken as an indication that the higher stimulation did produce normal responses and thus, that the actual stimulation strength at the neurons was lower than would be expected if resistance in the surface of the cultured slices was identical to that of acute slices. Goto et al (2010) concludes that extracellular conductivity can be a major factor of neuronal tissue resistance. It can be speculated that an increase in resistance could be caused by a change in surface morphology, possibly due to dead cells or a slightly different surface cell configuration compared to acute slices. This is also in line with detectable visual differences observed, suggesting that the slice surface has indeed changed. Due to the difficulties of accurately measuring tissue resistance (it would be a whole research project of its own) no investigation of the actual resistance in cultured slices was made.

Culture medium analysis

The rate of failed slices that never even made it to the electrophysiological recording was very high. Some of these slices were lost due to culture-external events out of the control of the experimenter. Other losses were likely affected by culture related problems. In some cultures the slices turned out to be very vulnerable to external violence and fell apart far more easily than they should have had they been "healthy". These disintegrated slices were not counted in the analysis. This means that the true success rate of the culturing might be lower than the already low rate of cases where a LTP response could be detected. In slices with preserved acute slice properties the success rate of LTP induction would be around 95% in younger animals and almost 100% for older animals for an experienced field recorder (according to the author's supervisor, Henrik Seth) and this includes discarding slices that do give a LTP response that is weaker than the slices of the same batch.

Due to the low success rate, resulting in few wave metric (wave data files recorded from the trials, analysed using the IGOR-pro software), the initial "pipe dream" of being able to analyse the wave metric data directly to obtain magnitude differences in response between populations had to be abandoned. It was also concluded that the data was insufficient to perform any multivariate correlation analysis. Still, it was necessary to perform some form of analysis on the data available to be able to answer the question of whether this method of hCSF cultivation of slices is viable. Instead of the initial plan, it was decided to use a binary classification of trial outcomes as either successful or failed. In the comparison of the culturing outcomes from hCSF cultures and NB cultures, it was decided to

use the EPSP response as a cut-off for what was condidered a successful trial. That is, only trials that produced a clearcly detectable EPSP recording were considered successful.

What to consider a trial in the analysis was considered extensively. Should the data be analysed using all slices as individual trials? Or would it be better to treat each culture well as a trial? Or to treat each individual animal as a trial? It was concluded that since the experiments used slices from the same animal for both hCSF and NB cultures, using animals as trials for the analysis would not be appropriate. It was also deemed likely that there would be a strong correlation of outcome for slices in the same well, further skewing a small data set if analysis would be made on individual slice level. Thus it was decided to analyse the data treating each culture well as a trial; considering each well containing at least 1 slice that produced an EPSP response or better (i.e. including LTP) as a success.

Since the outcome data was binary in nature, either being classified as a success or a failure at the EPSP level, the choice stood between Fisher's exact test and the chi-square test. It was deemed that since the sample size of NB slices was rather small (n=10), Fisher's exact test would be the better choice and the one trusted more (McDonald 2009). It does have some drawbacks, as exact tests in general tend to have a lower power, leading to less type 1 errors (false positives) than other tests, but also making them more prone to type 2 errors (missing an effect that is detectable). There are more accurate methods available for small samples. Lydersen et al (2009) describe and discuss several of them, but time-wise it was not an option to try to understand and apply them during the end of the project. A chi-square test was also performed, as it required no extra effort. No test detected any significant difference in outcome between the two groups (see Table 3).

Transfer well medium analysis

During the project, several aspects of the culturing protocol were discussed. One of those was the use of Gibco® HIBERNATE® Media (a cell culturing media available on the market) as a transfer well medium. Was this extra step really necessary? Or could it be replaced by the actual culture medium in the transfer well? As it would be an easy change to the existing protocol, it was decided mid-project, to try out a new culture preparation procedure; filling the transfer well with the culture medium to be used (hCSF or NB) rather than Hibernate.

From the analysis samples of the results of this change, NB was excluded; as no NB cultures were made at the early stage of the study during which hibernate was used. To evaluate the effect of the change in transfer medium, it was decided to use the culture well level, and the small sample size made Fisher's exact test appropriate (McDonald 2009). A chi-square comparison was also performed but done more because it took no extra effort than because it was really needed. Neither test was found to be significant at the 95% confidence level.

This gives an indication that the transfer well medium is either not an important factor, as both media gave low overall results, or that both media were inappropriate for use in the transfer well. Another possible reason might be that using the transfer well technique at all is a bad practise and should be abandoned in favour of direct transfer to the culture well. All of these options might be worth investigating further.

Analysis by age

It was deemed of interest to see if any difference based on the age of the animals used could be detected. There should be a considerable difference according to Stoppini et al (1991). The diagram in Figure 24 hints at presence of this age-effect on outcomes in this study.

It was decided to evaluate if any age effect could be detected at transfer well level, even if rat level might have been applicable here since both hCSF and NB cultures were included. The trials were binned age-wise as can be seen in Table 5. The majority of the rats were in the age groups of 6-10 and 11-15. Since there are more than two bins, it was decided that a chi-square test was appropriate for the hypothesis testing (McDonald, 2009). The performed analysis failed to show any significance (p-value: 0.251082) but as the test is unreliable and the data sparse, it is not possible to draw any reliable conclusions. While it would be interesting to investigate the age-effect further, it might not be the most pressing issue to focus on in upcoming trials.

Consecutive successful recordings from the same well

The data set obtained from the experiments contains recordings from more than one day from 20 of the 56 wells used in the project. Out of these, 4 wells showed successful slices at the EPSP level on more than one day. 3 additional slices showed EPSP responses on one of the days recording was made. This implies a correlation between slices in successful wells. It can be speculated that if slices do survive with intact properties the first days (during which, no recordings were made), there is an increased long-term viability and network activity. It also indicates that the majority of slices that did not respond to stimulation "died" early in the process. This could be due to several factors. The cutting is first and foremost causing severe trauma to the tissue, cutting several neurons and astrocytes (a type of glia cells), killing them. The trauma leads to increased astrocyte activity, inflammatory responses and activation of microglia, which has been associated with synaptic degeneration and (Kraft et al, 2016). Thus, if some alterations to the protocol would cause less trauma initially, or in some way enhance tissue recovery in the freshly cut slices, it could be a key to a more robust culturing protocol.

While the connection between initial survival and subsequent longevity was not investigated statistically in any way, the idea is intriguing. Since the time-cost for shorter screening trials of a few hours at the culture start is low, investigation of early slice survival and testing of a few different initial treatments is already under investigation by researchers at the department.

The acute slices

The results from the acute slices indicates that while the LTP responses are not perfect, they are still present and the field recordings performed by the author are not extensively flawed in general. This gives support to the idea that the majority of the lack of response in cultured slices is indeed due to the culturing process and not due to lack of experience and craftsmanship with the recordings or the slicing from the author's side.

Further discussion of all the experiments and speculation regarding methods to try out in the future

The results from the study and the far future

The overall low success rates of all the culturing experiments indicate that the culturing protocol and/or media used in this study are not reliable. Result from other studies, such as Gähwiler (1981), Stoppini et al (1991), Caeser and Aertsen (1991), Opitz-Araya and Barria (2011) and Carmeli et al. (2013) indicate that a higher success rate should be possible. However, it might be worth keeping in mind that success rates in some studies are for other methods, such as patch-clamping and staining with different dyes and might yield different results from extracellular field recordings. Use of cultured slices could be more reliable for those methods. Also, the number of slices used in studies that present positive results is small and there is not an abundance of studies using the method for follow-up trials. It could be speculated that the membrane culturing methods used by these groups might not be as robust as the published studies indicate and that there was selection bias in choice of samples to include in the articles.

Despite the lack of robustness experienced in this study, the method can produce results. The beginner's luck experiment at the beginning of the project suggests that it could well be worth the work to further investigate culturing, as the response quality was excellent compared to all other experiments conducted. If slices regularly could be kept alive for at least 4 days and still maintain that functionality in at least 80% of trials; that would make the method worthwhile to use for further studies in medical and other neuronal research. It could for example be used to expose the slices to hCSF from patients with Alzheimer's disease or long-term exposed to certain drugs at a well known concentration for a prolonged time.

It could also open a field of really long-term investigations of long-term potentiation and an opportunity to try out repeated LTP induction as described in a review regarding timing of learning, by Smolen, Zhang and Byrne (2016). Trying several LTP inductions with times as far between them as weeks is currently not possible given how short time acute slices survive, but could be of high interest to strengthen the scientific impact of learning studies conducted on students with cellular data pointing in the same direction. More data would give further opportunities to use mathematical models, such as those of Zhang et al (2012) to calculate optimal time intervals for repeated LTP induction and to predict other macro-scale phenomenons using microscale data. This could potentially be used to predict and design better rehabilitation protocols for stroke patients and better teaching protocols for school students. Possibly, cultured hippocampal slices could also provide a means to test the effect of different ion concentrations found in individuals diagnosed with neuropsychiatric diagnoses on LTP induction, uncovering clues about learning impairments and enhancements present in these groups.

In addition, since extracellular field recordings are time consuming, many of the slices cut for acute experiments are wasted as there is not enough time to use them all. A robust culturing method would present a practical way to conserve acute slices for work on later days, in effect reducing the number of animals needed for testing.

What should be done next?

In order to make the process more reliable, there is a need of extensive research into the factors that affect the survival of hippocampal tissue in vitro. Some studies, such as Caeser and Aertsen (1991) have focused on the morphology of cultured slices, while others, such as Opitz-Araya and Barria (2011) have explored the culturing process with respect to pH and osmolarity in artificial CSF. Possibly there is a lot of such research going on at different laboratories around the world, which is

indicated (author's speculation) by the steady trickle of articles published. On the other hand, if such research is being conducted at many laboratories, a lot of that research could be failed experiments that are impossible to publish, as there has been no explosion in publications utilizing hippocampal slice cultivation for further research.

Still, the successful trials gives hope to someday uncover a method for cultivation that is both simple and reliable. The work on this project has led to several ideas to follow up. As mentioned in Consecutive successful recordings from the same well, the idea to use fast screening experiments, letting slices incubate for a few hours before performing extracellular field recordings from them is already being investigated at the department of Neuroscience and Physiology and the University of Gothenburg.

Further investigation on culture media is also needed. In this study, the initial osmolarity of the hCSF used varied considerably. It could be of interest only use hCSF with an osmolarity close to 300 mOsmol/kg in future cultures. This could be achieved either by discarding hCSF not at the desired osmolarity, or by adding distilled water to hCSF with a slightly too high osmolarity.

Another factor that is of high interest is the handling of the slices prior to culturing. In the slicing protocol used for this study, the brain is kept in a cold (3°C) bath during the slicing process. This is done to minimize damage to the neurons from the trauma of cutting. Then the slices are placed in a bath at room temperature, which works really well for acute slices and that is (author's speculation) probably not that big of an issue when culturing either, but is that bath really needed? It could be worth trying to place the slices directly on the culturing membranes after slicing and start culturing immediately.

Temperature could also be a factor to investigate further. Is the shock of going from room temperature in the resting bath to the relatively high temperature of 37°C in the incubator causing any damage to the cells in the slice? It could be worth trying a lower temperature in the incubator during the first hours, gradually raising it instead of starting high. It has been proposed that therapeutic hypothermia (to intentionally lower the body temperature during surgery) helps preventing trauma from injuries and recent studies discussed in a review by Yokobori and Yokota (2016), indicate that the method works for specific kinds of brain trauma prevention. From that the step is not long to consider the possibility of inducing hypothermia in the slices at the start of culturing.

Another indication on synaptic plasticity caused by temperature can be found in hibernating animals. During hibernation, brain termperature can be lowered to values close to the freezing poing in some species and there is very low neuronal activity. Still, research such as that on hibernating ground squirrels from von der Ohe et al (2006) shows that when the animals wake up normal neuronal function and activity is quickly regained.

These temperature effects indicate that early cultivation at a few different temperatures, subsequently raising the temperature to 37°C could be of interest for future studies. Another wild idea to try out is to induce fever in the slices. Could culturing at to 38-39°C produce interesting and/or useful results?

Hopefully, the step is not that long until something is uncovered that makes culturing hippocampal slices in hCSF a routine procedure. That will enable the use in neuropathological research and possibly lead to new discoveries. Maybe even something that can be used to improve learning in the future, both in school situations and in life in general.

Conclusion

The research question to be answered by this thesis was: Are hippocampal slices from rat cultured in hCSF viable as a model for future research on human brain functions? It can be answered with a "no, but…", as the method tested yielded poor results overall, but also did produce some results indicating that it is indeed possible to use hCSF as a culture medium. This means that the method applied is not robust and not producing reliable results. The study reveals an increase in stability in pH and osmolarity in the cultures compared to a previous study at the same laboratory.

There are indications that slices that survive the first days of cultivation have a good chance of long-term preservation of acute-like properties during field recordings, and that a major problem in obtaining robust results could be that slices die early during cultivation. This raises further questions that need to be followed up regarding how to make the slices survive the first few hours and follow up studies should focus on the transition phase between the slicing and the cultures and on the initial culturing period.

Afterword: Informal free-thinking from the author regarding the connection of neuroscience and learning

At the onset of this master's project, I had no idea where the journey would take me. I was looking for a project in neuroscience that could also be connected to the main focus of my master's programme in learning and leadership. While the research work in this masters project might not in itself be of any direct use for teaching or didactics in any foreseeable future, there are more connections between the fields than I first thought.

The extensive reading of articles in the field of neuroscience and related fields have really transformed my way of seeing the learning process. So much more of it can be explained on a molecular or cellular level than I was aware of, even if we still know but a fraction of the mechanisms behind how the mind is shaped by the neurons that build up the central nervous system. It is a both fascinating and extremely humbling thought that despite the extensive knowledge amassed in the field of neuroscience, we have just barely begun to understand what future generations might take for granted.

The emerging understanding of how memories form revealed by optogenetic studies such as that of Takagi et al (2015) and the different investigations into repeated LTP induction discussed by Smolen, Zhang and Byrne (2016) are connected to the research the extracellular recordings I have made during the project. They all involve the same mechanisms of strengthening of the synapses in neurons. Another connection that is not as apparent is the possible of future applications of a robust culturing protocol. It could be used to provide more data to fit computer models such as those of Zhang et al (2012) to calculate optimal time intervals for repeated LTP, strengthening certain engrams (circuits of neurons that represent a specific memory) and also to test predictions made by such models. Possibly, cultured hippocampal slices could also provide a means to test the effect of different ion concentrations found in individuals diagnosed with neuropsychiatric diagnoses on LTP induction, uncovering clues about learning impairments and enhancements present in these groups.

The more I learn in the field of neuroscience, the more convinced I get that it is important that future didactic researchers initiate an extensive collaboration with neuroscientists and researchers in psychology. The fields are far more connected, via the neurons in our brains and the networks they form, and how those networks shape our behaviour, than has previously been acknowledged within the respective disciplines.

For my own part, I have taken a few steps along this interdisciplinary path, as the thesis work has also offered concrete practise and work in didactics. A secondary objective of the thesis was the work of producing material for use in the education of to-be teachers in mathematics and a scientific subject studying the first year at the Learning and Leadership (MPLOL) master's programme at Chalmers University of Technology. The material produced and the work conducted is presented in the appendix. I initially thought that that would be the major didactical challenge of this master's project. I was very, very wrong on that account. The major challenge has instead proved to be to write this report and to prepare the presentation. The reason for this is that it has to be possible for anyone with just a general background in any science field to read and understand my report. It is not intended for colleagues in the field the work was conducted in, but rather aimed at the varied audience of the the unique master's program in Learning and Leadership. This has forced me to go through every piece more than once to ensure that it is comprehensible for anyone, and accounts for a lot of the pages in this report. I can only hope that I have managed to step up to the challenge in a satisfactory way, and that you when you read this feels that you have gained insights in a field that is highly fascinating and more closely connected to anything we do when we teach others than might be apparent.

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Appendix

Appendix A: Additional data tables and analysis

pH screening trial ANOVA

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
hCSF	5	39,96	7,992	0,10612
hepes 1mM	5	39,23	7,846	0,02748
hepes 5mM	5	38,66	7,732	0,05372
hepes 10mM	5	38,65	7,73	0,10415

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0,23002	3	0,076673333	1,052229503	0,396675	3,238872
Within Groups	1,16588	16	0,0728675			
Total	1,3959	19				

Anova: Single Factor

Table 7. ANOVA output for the initial screening of pH with hepes in the hCSF. No significance was obtained, though the average of hCSF is a little higher than the samples containing hepes additions.

Table of data for the investigation of hepes addition as extra buffer

Trial ID	time (h)	pH (hCSF)	pH (hCSF+1mM hepes)
Trials with only hCSF and with added mM hepes			
2016-02-22-1-p7	102	7,29	7,48
2016-02-22-2-p7	101	7,31	7,48
2016-03-01-1-p15	72	7,72	7,74
2016-03-01-1-p15	72	7,52	7,81
2016-03-01-1-p15	146	7,62	7,6
2016-03-01-1-p15	146	7,67	7,55
2016-03-02-1-p16	122	7,25	7,53
2016-03-02-1-p16	122	7,2	7,43
More data from trials with only hCSF			
2016-03-09-1-p23	125	7,22	
2016-03-11-1-p25	123	7,4	
2016-03-11-1-p25	123	7,36	
2016-03-18-1-p10	98	7,46	
2016-03-18-1-p10	98	7,51	
2016-03-21-1-p8	75	7,3	
2016-03-21-1-p8	75	7,27	
Extra trials with no culture			
2016-03-17-1-hepestest	27	7,3	7,33
2016-03-17-1-hepestest	27	7,36	7,22
2016-03-17-1-hepestest	96	7,38	7,5
2016-03-17-1-hepestest	96	7,58	7,6
2016-03-17-1-hepestest	121	7,62	7,59
2016-03-17-1-hepestest	121	7,62	7,6
2016-03-17-2-hepestest	27	7,46	7,35
2016-03-17-2-hepestest	27	7,52	7,5
2016-03-17-2-hepestest	121	7,57	7,53
2016-03-17-2-hepestest	121	7,38	7,36

Table 8: Full data from the trials to determine if adding 1mM hepes has enough buffering effect to warrant the addition to hCSF culture medium.Included is all data from trials conducted up to 2016-03-25.

Hepes investigation ANOVAs

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	10	74,74	7,474	0,018493
Column 2	10	74,63	7,463	0,013557

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0,000605	1	0,000605	0,037754	0,848115	4,413873
Within Groups	0,28845	18	0,016025			
Total	0,289055	19				

Table 9 ANOVA for the full trial hepes including only the data from concurrent cultures of hCSF and hCSF+1 mM hepes.

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
hCSF	18	134,64	7,48	0,035647
hCSF+hepes	18	135,25	7,513889	0,017943

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0,010336	1	0,010336	0,385749	0,538684	4,130018
Within Groups	0,911028	34	0,026795			
Total	0,921364	35				

Table 10. ANOVA for the full trial hepes including all hCSF data from pure hCSF cultures up to

Table of osmolarity with different media

culture time	hCSF	hCSF +1mM hepes	NB
0	468	377	302
0	266		252
0	292		254
0	299		258
0	296		251
0	287		312
0	282		260
0	285		262
0	302		250
0	261		
0	374		
0	205		
0	205		
29	336		
29			291
50	351		309
51	360		
51	374		
52	345		301
52	328		306
68	368		
68	405		
71	315		
71			306
72	346	337	349
72	350	325	
72	358		
74	249		
74	248		
75	261		
75	280		
76			323
98	298		
98	290		
98	398		
98	427		
102	381	385	
102	374	336	
120	427		382
122	435	452	
122	361	363	
122	264		
122	287		
123	286		
123	279		
124	454		391
125	376		
125	393		
146	378	380	
146	368	372	

Table 11. Table of osmolarity recordings for the different media.

Full table of raw data

Trial ID	age	transfer well medium	medium	well	number in well	days in culture	slicer	LTP	EPSP	Volley	wasted slice	left stim	right stim	R	EPSP magnitude	LTP magnitude	incubator
2016-02-05-1-p27-practise	27	hibernate	hCSF	1	1	4	5	yes	yes	yes	no	320	320	NA	0,940082	1,366867	old
2016-02-11-1-p9-practise	9	hibernate	hCSF	2	1	4	1	NA	NA	NA	yes	NA	NA	2,6	NA	NA	old
2016-02-11-1-p9-practise	9	hibernate	hCSF	2	2	4	1	NA	no	yes	no	300	300	2,6	1,245182	1,06056	old
2016-02-17-1-p15-practise	15	hibernate	hCSF	3	1	2	3	no	no	no	no	320	320	NA	NA	NA	old
2016-02-17-1-p15-practise	15	hibernate	hCSF	3	2	2	3	no	no	no	no	320	320	NA	NA	NA	old
2016-02-17-1-p15-practise	15	hibernate	hCSF	3	3	2	3	no	no	no	no	320	320	NA	NA	NA	old
2016-02-17-1-p15-practise	15	hibernate	hCSF	3	4	2	3	no	no	no	no	320	320	NA	NA	NA	old
2016-02-22-1-p7-practise	7	hibernate	hCSF	4	1	1	1	no	no	no	no	320	320	1,7	NA	NA	old
2016-02-22-1-p7-practise	7	hibernate	hCSF	4	2	2	1	NA	NA	NA	yes	NA	NA	NA	NA	NA	old
2016-02-22-1-p7-practise	7	hibernate	hCSF	4	3	4	1	NA	NA	NA	yes	NA	NA	NA	NA	NA	old
2016-02-22-1-p7-practise	7	hibernate	hCSF+hepes	5	1	1	1	no	NA	NA	yes	NA	NA	NA	NA	NA	old
2016-02-22-1-p7-practise	7	hibernate	hCSF+hepes	5	2	2	1	yes	yes	yes	no	200	200	1,7	NA	NA	old
2016-02-22-1-p7-practise	7	hibernate	hCSF+hepes	5	3	4	1	NA	NA	NA	yes	NA	NA	NA	NA	NA	old
2016-02-22-2-p7-practise	7	hibernate	hCSF	6	1	1	3	no	no	no	no	320	320	NA	NA	NA	old
2016-02-22-2-p7-practise	7	hibernate	hCSF	6	2	2	3	yes	yes	yes	no	100	230	1,7	2,151203	6,443892	old
2016-02-22-2-p7-practise	7	hibernate	hCSF	6	3	4	3	NA	NA	NA	yes	NA	NA	NA	NA	NA	old
2016-02-22-2-p7-practise	7	hibernate	hCSF+hepes	7	1	1	3	no	no	no	no	320	320	NA	NA	NA	old
2016-02-22-2-p7-practise	7	hibernate	hCSF+hepes	7	2	2	3	no	no	yes	no	320	320	1,7		NA	old
2016-02-22-2-p7-practise	7	hibernate	hCSF+hepes	7	3	4	3	NA	NA	NA	yes	NA	NA	NA	NA	NA	old
2016-03-01-1-p15	15	hibernate	hCSF	8	1	1	1	NA	NA	NA	yes	NA	NA	NA	NA	NA	old
2016-03-01-1-p15	15	hibernate	hCSF	8	2	2	1	NA	NA	NA	yes	NA	NA	NA	NA	NA	old
2016-03-01-1-p15	15	hibernate	hCSF	8	3	6	1	NA	NA	NA	yes	NA	NA	NA	NA	NA	old
2016-03-01-1-p15	15	hibernate	hCSF+hepes	9	1	1	1	NA	NA	NA	yes	NA	NA	NA	NA	NA	old
2016-03-01-1-p15	15	hibernate	hCSF+hepes	9	2	6	1	NA	NA	NA	yes	NA	NA	NA	NA	NA	old
2016-03-01-1-p15	15	hibernate	hCSF+hepes	9	3	6	1	NA	NA	NA	yes	NA	NA	NA	NA	NA	old
2016-03-01-1-p15	15	hibernate	hCSF	10	1	6	1	NA	NA	NA	yes	NA	NA	NA	NA	NA	old
2016-03-01-1-p15	15	hibernate	hCSF	10	2	6	1	no	no	yes	no	140	210	1,8	NA	NA	old
2016-03-01-1-p15	15	hibernate	hCSF	10	3	6	1	NA	NA	NA	yes	NA	NA	NA	NA	NA	old
2016-03-01-1-p15	15	hibernate	hCSF+hepes	11	1	6	1	NA	NA	NA	yes	NA	NA	NA	NA	NA	old
2016-03-01-1-p15	15	hibernate	hCSF+hepes	11	2	6	1	NA	NA	NA	yes	NA	NA	NA	NA	NA	old
2016-03-01-1-p15	15	hibernate	hCSF+hepes	11	3	6	1	NA	NA	NA	yes	NA	NA	NA	NA	NA	old
2016-03-02-1-p16	16	hibernate	hCSF	12	1	6	1	NA	NA	NA	yes	NA	NA	NA	NA	NA	old
2016-03-02-1-p16	16	hibernate	hCSF	12	2	6	1	NA	NA	NA	yes	NA	NA	NA	NA	NA	old
2016-03-02-1-p16	16	hibernate	hCSF	12	3	6	1	NA	NA	NA	yes	NA	NA	NA	NA	NA	old
2016-03-02-1-p16	16	hibernate	hCSF+hepes	13	1	6	1	NA	NA	NA	yes	NA	NA	NA	NA	NA	old
2016-03-02-1-p16	16	hibernate	hCSF+hepes	13	2	6	1	NA	NA	NA	yes	NA	NA	NA	NA	NA	old
2016-03-02-1-p16	16	hibernate	hCSF+hepes	13	3	6	1	NA	NA	NA	yes	NA	NA	NA	NA	NA	old
2016-03-02-1-p16	16	hibernate	hCSF	14	1	6	1	NA	NA	NA	yes	NA	NA	NA	NA	NA	old
2016-03-02-1-p16	16	hibernate	hCSF	14	2	6	1	NA	NA	NA	yes	NA	NA	NA	NA	NA	old
2016-03-02-1-p16	16	hibernate	hCSF	14	3	6	1	NA	NA	NA	yes	NA	NA	NA	NA	NA	old
2016-03-02-1-p16	16	hibernate	hCSF+hepes	15	1	6	1	NA	NA	NA	yes	NA	NA	NA	NA	NA	old
2016-03-02-1-p16	16	hibernate	hCSF+hepes	15	2	6	1	NA	NA	NA	yes	NA	NA	NA	NA	NA	old
2016-03-02-1-p16	16	hibernate	hCSF+hepes	15	3	6	1	NA	NA	NA	yes	NA	NA	NA	NA	NA	old
2016-03-29-1-p23	23	hibernate	hCSF	16	1	1	1	no	no	yes	no	280	280	5,3	NA	NA	old
2016-03-09-1-p23	23	hibernate	hCSF	16	2	1	1	NA	NA	NA	yes	NA	NA	3,5	NA	NA	old
2016-03-29-1-p23	23	hibernate	hCSF	16	3	2	1	no	no	no	no	290	100	1,8	NA	NA	old
2016-03-09-1-p23	23	hibernate	hCSF	16	4	2	1	no	yes	yes	no	290	100	3,8	1,114324	NA	old
2016-03-29-1-p23	23	hibernate	hCSF	17	1	5	1	no	no	no	no	320	320	3,3	NA	NA	old
2016-03-09-1-p23	23	hibernate	hCSF	17	2	5	1	no	no	no	no	320	320	3,8	NA	NA	old
2016-03-29-1-p23	23	hibernate	hCSF	17	3	5	1	no	no	no	no	320	320	3,8	NA	NA	old
2016-03-09-1-p23	23	hibernate	hCSF	17	4	5	1	NA	NA	NA	yes	NA	NA	NA	NA	NA	old
2016-03-11-1-p25	25	hibernate	hCSF	18	1	5	1	no	no	no	no	320	320	NA	NA	NA	old
2016-03-11-1-p25	25	hibernate	hCSF	18	2	5	1	no	no	no	no	320	320	NA	NA	NA	old
2016-03-11-1-p25	25	hibernate	hCSF	18	3	5	1	no	no	no	no	320	320	NA	NA	NA	old
2016-03-11-1-p25	25	hibernate	hCSF	19	1	3	1	no	no	no	no	320	320	5	NA	NA	old
2016-03-11-1-p25	25	hibernate	hCSF	19	2	3	1	NA	NA	NA	yes	NA	NA	3,5	NA	NA	old
2016-03-15-1-p15	15	hibernate	hCSF	20	1	2	1	no	no	no	no	320	320	3,2	NA	NA	old
2016-03-15-1-p15	15	hibernate	hCSF	20	2	2	1	NA	NA	NA	yes	yes	yes	NA	NA	NA	old
2016-03-15-1-p15	15	hibernate	hCSF	20	3	2	1	NA	NA	NA	yes	yes	yes	NA	NA	NA	old
2016-03-15-1-p15	15	hibernate	hCSF	21	1	2	1	NA	NA	NA	yes	yes	yes	NA	NA	NA	old
2016-03-15-1-p15	15	hibernate	hCSF	21	2	2	1	no	no	no	no	320	320	3,2	NA	NA	old
2016-03-15-1-p15	15	hibernate	hCSF	21	3	2	1	no	no	no	no	320	320	3,2	NA	NA	old
2016-03-15-2-p15	15	hibernate	hCSF	22	1	2	1	no	no	no	no	320	320	3,2	NA	NA	old
2016-03-15-2-p15	15	hibernate	hCSF	22	2	2	1	no	no	no	no	320	320	3,2	NA	NA	old
2016-03-15-2-p15	15	hibernate	hCSF	23	3	2	1	no	no	no	no	320	320	NA	NA	NA	old
2016-03-15-2-p15	15	hibernate	hCSF	23	1	2	1	NA	NA	NA	no	NA	NA	NA	NA	NA	old
2016-03-15-2-p15	15	hibernate	hCSF	23	2	2	1	NA	NA	NA	no	NA	NA	NA	NA	NA	old
2016-03-18-1-p10	10	hibernate	hCSF	24	1	3	1	no	no	no	no	320	320	2,4	NA	NA	old
2016-03-18-1-p10	10	hibernate	hCSF	24	2	3	1	no	yes	yes	no	210	320	1,2	0,671987	0,7397	old
2016-03-18-1-p10	10	hibernate	hCSF	24	3	4	1	no	yes	yes	no	320	320	2,3	1,378674	0,703654	old
2016-03-18-1-p10	10	hibernate	hCSF	24	4	4	1	no	no	yes	no	240	270	2,3	1,723598	0,823265	old
2016-03-18-1-p10	10	hibernate	hCSF	25	1	3	1	no	no	no	no	320	320	2,4	NA	NA	old
2016-03-18-1-p10	10	hibernate	hCSF	25	2	4	1	no	no	no	no	320	320	2,3	NA	NA	old
2016-03-18-1-p10	10	hibernate	hCSF	25	3	4	1	no	no	no	no	320	320	2,3	NA	NA	old
2016-03-18-1-p10	10	hibernate	hCSF	25	4	4	1	NA	NA	NA	yes	NA	NA	NA	NA	NA	old
2016-03-21-1-p8	8	hCSF	hCSF	26	1	3	1	NA	NA	NA	yes	NA	NA	2,2	NA	NA	old
2016-03-21-1-p8	8	hCSF	hCSF	26	2	3	1	no	no	yes	no	140	140	2,2	1,061203	0,225849	old
2016-03-21-1-p8	8	hCSF	hCSF	26	3	3	1	no	yes	yes	no	280	240	2,9	2,888065	0,244497	old
2016-03-21-1-p8	8	hCSF	hCSF	27	1	2	1	no	no	yes	no	320	160	7,4	-0,55997	0,436774	old

2016-03-21-1-p8	8	hCSF	hCSF	27	2	3	1	NA	NA	NA	yes	NA	NA	2,9	NA	NA	old
2016-03-21-1-p8	8	hCSF	hCSF	27	3	3	1	no	yes	yes	no	140	240	2,9	0,327702	-7,069521	old
2016-03-29-1-p8	8	hCSF	hCSF	28	1	2	1	no	no	no	no	320	320	3	NA	NA	old
2016-03-29-1-p8	8	hCSF	hCSF	28	2	2	1	no	no	yes	no	320	320	4,5	NA	NA	old
2016-03-29-1-p8	8	hCSF	hCSF	28	3	2	1	no	no	no	no	320	320	4,5	NA	NA	old
2016-03-29-1-p8	8	hCSF	hCSF	29	1	2	1	no	yes	yes	no	70	NA	3,2	1,377381	1,324862	old
2016-03-29-1-p8	8	hCSF	hCSF	29	2	3	1	no	yes	yes	no	320	240	3,1	16,59325	43,81799	old
2016-03-29-1-p8	8	hCSF	hCSF	29	3	3	1	no	no	no	no	320	320	5,2	NA	NA	old
2016-03-30-1-p9	9	hCSF	hCSF	30	1	2	1	no	no	no	no	320	320	4,9	NA	NA	old
2016-03-30-1-p9	9	hCSF	hCSF	30	2	5	1	NA	NA	NA	yes	NA	NA	NA	NA	NA	old
2016-03-30-1-p9	9	hCSF	hCSF	30	3	5	1	NA	NA	NA	yes	NA	NA	NA	NA	NA	old
2016-03-30-1-p9	9	hCSF	hCSF	31	1	2	1	no	no	no	no	320	320	4,9	NA	NA	old
2016-03-30-1-p9	9	hCSF	hCSF	31	2	5	1	NA	NA	NA	yes	NA	NA	NA	NA	NA	old
2016-03-30-1-p9	9	hCSF	hCSF	31	3	5	1	NA	NA	NA	yes	NA	NA	NA	NA	NA	old
2016-04-05-1-p8x2	8	hCSF	hCSF	32	1	2	1	no	yes	yes	no	24	24	2,8	1,270606	0,710772	old
2016-04-05-1-p8x2	8	hCSF	hCSF	32	2	4	1	no	yes	yes	no	24	28	2,8	0,566554	0,952285	old
2016-04-05-1-p8x2	8	hCSF	hCSF	32	3	4	1	NA	NA	NA	yes	NA	NA	NA	NA	NA	old
2016-04-05-1-p8x2	8	hCSF	hCSF	33	1	3	2	yes	yes	yes	no	320	240	2,8	6,21992	1,547906	old
2016-04-05-1-p8x2	8	hCSF	hCSF	33	2	4	2	no	yes	yes	no	20	28	NA	0,566554	0,952285	old
2016-04-05-1-p8x2	8	hCSF	hCSF	33	3	4	2	NA	NA	NA	yes	NA	NA	NA	NA	NA	old
2016-04-08-1-p12	12	hCSF	hCSF	34	1	3	4	no	no	no	no	320	320	2,9	NA	NA	old
2016-04-08-1-p12	12	hCSF	hCSF	34	2	3	4	no	no	no	no	320	320	2,9	NA	NA	old
2016-04-08-1-p12	12	hCSF	hCSF	34	3	3	4	no	no	no	no	320	320	2,9	NA	NA	old
2016-04-08-1-p12	12	hCSF	hCSF	35	1	3	4	no	no	no	no	320	320	4	NA	NA	old
2016-04-08-1-p12	12	hCSF	hCSF	35	2	3	4	no	no	no	no	320	320	2,1	NA	NA	old
2016-04-08-1-p12	12	hCSF	hCSF	35	3	4	4	no	no	no	no	320	320	6,4	NA	NA	old
2016-04-12-1-p15	15	hCSF	hCSF	36	1	4	1	no	no	no	no	320	320	1,1	NA	NA	old
2016-04-12-1-p15	15	hCSF	hCSF	36	2	4	1	no	no	no	no	320	320	1,1	NA	NA	old
2016-04-12-1-p15	15	hCSF	hCSF	36	3	5	1	NA	NA	NA	yes	NA	NA	NA	NA	NA	old
2016-04-12-1-p15	15	hCSF	hCSF	37	1	4	1	NA	NA	NA	yes	NA	NA	NA	NA	NA	old
2016-04-12-1-p15	15	hCSF	hCSF	37	2	4	1	NA	NA	NA	yes	NA	NA	NA	NA	NA	old
2016-04-12-1-p15	15	hCSF	hCSF	37	3	4	1	no	no	no	no	320	320	1,1	NA	NA	old
2016-04-14-1-p11	11	hCSF	hCSF	38	1	4	1	no	yes	yes	no	32	24	3,2	7,456966	0,281818	old
2016-04-14-1-p11	11	hCSF	hCSF	38	2	4	1	no	yes	yes	no	24	24	2,1	1,083212	0,952038	old
2016-04-14-1-p11	11	hCSF	hCSF	38	3	5	1	no	no	no	no	320	320	3,5	NA	NA	old
2016-04-14-1-p11	11	NB	NB	39	1	4	1	no	yes	yes	no	240	270	3,2	9,500599	3,9111	old
2016-04-14-1-p11	11	NB	NB	39	2	4	1	no	yes	yes	no	20	16	1,9	4,467958	1,552263	old
2016-04-14-1-p11	11	NB	NB	39	3	4	1	no	yes	yes	no	18	12	1,7	0,967703	0,767261	old
2016-04-19-1-p15	15	hCSF	hCSF	40	1	2	1	NA	NA	NA	yes	NA	NA	NA	NA	NA	old
2016-04-19-1-p15	15	hCSF	hCSF	40	2	2	1	no	no	no	no	320	320	3,8	NA	NA	old
2016-04-19-1-p15	15	hCSF	hCSF	40	3	2	1	no	no	no	no	320	320	3,8	NA	NA	old
2016-04-19-1-p15	15	NB	NB	41	1	2	1	no	no	no	no	320	320	3,8	NA	NA	old
2016-04-19-1-p15	15	NB	NB	41	2	2	1	no	no	yes	no	320	320	3,8	-1,50933	2,727894	old
2016-04-19-1-p15	15	NB	NB	41	3	2	1	no	no	no	no	320	320	3,8	NA	NA	old
2016-04-22-1-p12	12	hCSF	hCSF	42	1	4	1	no	no	no	no	320	320	4	NA	NA	old
2016-04-22-1-p12	12	hCSF	hCSF	42	2	4	1	no	no	no	no	320	320	2,3	NA	NA	old
2016-04-22-1-p12	12	hCSF	hCSF	42	3	5	1	no	no	no	no	320	320	1	NA	NA	old
2016-04-22-1-p12	12	hCSF	hCSF	42	4	5	1	no	no	no	no	320	320	1	NA	NA	old
2016-04-22-1-p12	12	NB	NB	43	1	4	1	no	no	no	no	320	320	2,3	NA	NA	old
2016-04-22-1-p12	12	NB	NB	43	2	4	1	no	yes	yes	no	32	90	2,3	NA	NA	old
2016-04-22-1-p12	12	NB	NB	43	3	4	1	no	yes	yes	no	17	21	2,3	NA	NA	old
2016-04-22-1-p12	12	NB	NB	43	4	5	1	no	no	no	no	320	320	1	NA	NA	old
2016-04-26-1-p16	16	hCSF	hCSF	44	1	2	1	no	no	no	no	320	320	5,8	NA	NA	new
2016-04-26-1-p16	16	hCSF	hCSF	44	2	2	1	no	no	no	no	320	320	4,4	NA	NA	new
2016-04-26-1-p16	16	hCSF	hCSF	44	3	2	1	no	no	no	no	320	320	1,8	NA	NA	new
2016-04-26-1-p16	16	NB	NB	45	1	2	1	NA	NA	NA	yes	NA	NA	5,8	NA	NA	new
2016-04-26-1-p16	16	NB	NB	45	2	2	1	no	no	no	no	320	320	5,8	NA	NA	new
2016-04-26-1-p16	16	NB	NB	45	3	2	1	no	no	no	no	320	320	1,8	NA	NA	new
2016-04-29-1-p10	10	hCSF	hCSF	46	1	3	1	no	no	no	no	320	320	1,9	NA	NA	new
2016-04-29-1-p10	10	hCSF	hCSF	46	2	3	1	no	no	no	no	320	320	1,9	NA	NA	new
2016-04-29-1-p10	10	NB	NB	47	1	3	1	no	yes	yes	no	240	240	1,9	-17,4573	-2,611602	new
2016-04-29-1-p10	10	NB	NB	47	2	3	1	no	yes	yes	no	240	240	1,9	0,92682	0,856815	new
2016-04-29-1-p10	10	NB	NB	47	3	3	1	no	yes	yes	no	120	100	1,9	2,275644	0,424834	new
2016-05-03-1-p8	8	hCSF	hCSF	48	1	2	1	no	yes	yes	no	160	140	1,5	0,731411	0,793841	new
2016-05-03-1-p8	8	hCSF	hCSF	48	2	2	1	no	no	no	no	320	320	2,4	NA	NA	new
2016-05-03-1-p8	8	hCSF	hCSF	48	3	2	1	no	yes	yes	no	240	240	2,4	0,896205	0,624161	new
2016-05-03-1-p8	8	hCSF	hCSF	48	4	2	1	NA	NA	NA	yes	NA	NA	NA	NA	NA	new
2016-05-03-1-p8	8	NB	NB	49	1	2	1	NA	NA	NA	yes	NA	NA	NA	NA	NA	new
2016-05-03-1-p8	8	NB	NB	49	2	2	1	NA	NA	NA	yes	NA	NA	NA	NA	NA	new
2016-05-03-1-p8	8	NB	NB	49	3	2	1	no	yes	yes	no	32	22	2,4	0,924046	0,619831	new
2016-05-03-1-p8	8	NB	NB	49	4	2	1	no	no	yes	no	240	190	2,4	-3,78812	0,943065	new
20160503-2-p22	22	hCSF	hCSF	50	1	3	1	no	no	no	no	320	320	3,5	NA	NA	new
20160503-2-p22	22	hCSF	hCSF	50	2	3	1	no	no	no	no	320	320	3,5	NA	NA	new
20160503-2-p22	22	NB	NB	51	1	3	1	no	no	no	no	320	320	3,5	NA	NA	new
20160503-2-p22	22	NB	NB	51	2	3	1	no	no	no	no	320	320	3,5	NA	NA	new
20160503-2-p22	22	NB	NB	51	3	3	1	no	no	no	no	320	320	3,5	NA	NA	new
2016-05-04-1-p16	16	hCSF	hCSF	52	1	2	1	no	no	no	no	320	320	NA	NA	NA	new
2016-05-04-1-p16	16	hCSF	hCSF	52	2	2	1	no	no	no	no	320	320	NA	NA	NA	new
2016-05-04-1-p16	16	hCSF	hCSF	52	3	2	1	NA	NA	NA	yes	NA	NA	NA	NA	NA	new
2016-05-04-1-p16	16	hCSF	hCSF	52	4	2	1	no	no	no	no	320	320	NA	NA	NA	new
2016-05-04-1-p16	16	hCSF	hCSF	52	5	2	1	no	no	no	no	320	320	NA	NA	NA	new
2016-05-04-1-p16	16	NB	NB	53	1	2	1	NA	NA	NA	yes	NA	NA	NA	NA	NA	new
2016-05-04-1-p16	16	NB	NB	53	2	2	1	no	no	no	no	320	320	NA	NA	NA	new
2016-05-04-1-p16	16	NB	NB	53	3	2	1	no	no	no	no	320	320	NA	NA	NA	new
2016-05-04-1-p16	16	NB	NB	53	4	2	1	NA	NA	NA	yes	NA	NA	NA	NA	NA	new
2016-05-04-2-p23	23	hCSF	hCSF	54	1	2	1	no	no	no	no	320	320	NA	NA	NA	new

2016-05-04-2-p23	23	hCSF	hCSF	54	2	2	1	no	no	no	no	320	320	NA	NA	NA	new
2016-05-04-2-p23	23	hCSF	hCSF	54	3	2	1	no	no	no	no	320	320	NA	NA	NA	new
2016-05-04-2-p23	23	hCSF	hCSF	54	4	2	1	NA	NA	NA	yes	NA	NA	NA	NA	NA	new
2016-05-04-2-p23	23	hCSF	hCSF	54	5	2	1	NA	NA	NA	yes	NA	NA	NA	NA	NA	new
2016-05-04-2-p23	23	NB	NB	55	1	2	1	NA	NA	NA	yes	NA	NA	NA	NA	NA	new
2016-05-04-2-p23	23	NB	NB	55	2	2	1	no	no	no	no	320	320	NA	NA	NA	new
2016-05-04-2-p23	23	NB	NB	55	3	2	1	no	no	no	no	320	320	NA	NA	NA	new
2016-05-04-2-p23	23	NB	NB	55	4	2	1	no	no	no	no	320	320	NA	NA	NA	new
2016-05-04-2-p23	23	NB	NB	55	5	2	1	NA	NA	NA	yes	NA	NA	NA	NA	NA	new
2016-05-06-1-p11	11	NB	NB	56	1	3	2	no	yes	yes	no	16	19	2,1	3,114082	0,626859	new
2016-05-06-1-p11	11	NB	NB	56	2	3	2	no	yes	yes	no	320	240	2,1	0,87724	1,132447	new
2016-05-06-1-p11	11	NB	NB	56	3	3	2	no	yes	yes	no	140	120	2,1	0,838873	1,178063	new

Table 12: Table of raw data. Some of it was used for calculations under results.

Appendix B: Direct didactical part of this masters project in neuroscience

Below follows an educational article that was prepared for use as extra material in the course Analyzing learning (MVE380) at Chalmers University of Technology given the fourth reading period during spring 2016. The intention of the material was to broaden the students perspectives on modern learning theories by making some results from frontline neurological research accesible without a background in neuroscience. It was supplied along with a discussion task intended for the lesson a week after the initial reading of the article. The discussion task is formulated as a case and is in swedish, as the course was taught in swedish. The article and discussion task were used as intended during the course and the author was presented observing the class.

The article and task were slightly edited after their use. No formal evaluation of the work was done, but some informal fast evaluation was conducted by the author together with the responsible teacher of the course. After the article a reflection from the author regarding the outcome is supplied.

Andreas Gustafsson, Spring 2016,
Didactical part of a Masters Thesis at the MPLOL masters programme
Chalmers University of Technology.
Intended for use as extra material in teaching modern aspects of learning theories.

Recent discoveries in neuroscience related to modern theories and methods of learning

Recent theories of learning include ***spaced learning*** and ***interleaving*** (Bjork, 2016). That the method of spaced learning is superior to the method of massed learning for a variety of learning situations is well established in psychologic and didactic research. However, the underlying mechanisms has until recently been an unmapped area. Novel methods of biochemistry, neurobiology and physics are now showing promising results in uncharting the mechanisms on a molecular and cellular level. In addition, computational models show some promise for further improving the learning protocols tested by psychologists and researchers in didactics. This text attempts to explain a few recent (at the time of writing) discoveries in neuroscience, and how they can help us understand the nature of learning better as teachers of students in the field of science.

Some basics on neurons and terms used in neuroscience
In order to understand the results discussed below it is necessary to have some background information on how neurons intact and some general outlines of the molecular processes involved, even if the details of every process is far too complex to discuss here.

Neurons (nerve cells) are composed of a cell body and protruding extensions. These extensions come in two forms. One is the ***axon***, which is a prolonged shaft along which electrical signals in the cells travel (relatively) uninterrupted. The axon on some neurons is coated in an insulating layer called myelin, produced by suppoert cells around the neurons. This layer improves the speed at which the nerve signal can travel. The other form of extensions are called ***dendrites***, and are protruding in a tree-like structure from the main cell body. On the dendrites smaller protrusions that are called ***dendritic spines*** are located. On the surface of these spines, the synapses are located.

A ***synapse*** is a small patch on the spine surface that is especially rich in chemoreceptor proteins and other compounds used for chemical signalling between two neurons. The synapse acts as a relay-station for messages between the cells. The synapse receiving a signal from it's neighbour gives rise to an ***action potential*** (an electrical signal generated chemically), that travels along the neuron until it reaches another synapse connected to the next neuron to send the signal to.

There are several types of synapses, each using a different mix of signal molecules to communicate with the neighbouring cells, and neurons in the brain have thousands of synapses connected to many other neurons, both very close and further away from the cell itself, connecting different parts of the brain with each other.

Not every input signal a neuron receives from its neighbouring cells is sent further in the network it is a part of. There is a threshold for when a signal is large enough to be propagated in a neuron from the synapses. This threshhold can be adjusted up or down, termed ***excitation*** and ***inhibition*** respectively, by several different mechanisms. Some adjustments are fast and short lasting while others are slower to activate and can last for days or even years.

The longer lasting changes are thought to be important for memory formation and deletion. ***Long Term Potentiation, LTP,*** is a lasting up-regulation of synapse strength. The same magnitude of input signal gives rise to a stronger electrical output signal at the synapse in response. The opposing effect, a down-regulation of the synaptic strength, meaning that the same magnitude of input signal gives a weaker response at the synapse, is called ***Long Term Depression, LTD***.

Recent results from LTP studies

The biochemical mechanisms underlying the LTP and LTD changes are not fully understood today, but some insight has been gained in recent years. Short high frequency electrical signals, typical of those occurring in the brain during memory activity, have been known to induce LTP in neurons for a long time. However, most experiments have only focused on a single such burst and the following increase. Recently several studies focusing on the effects of more than one such burst given with a resting period of varying length between them has been conducted.

Smolen, Zhang and Byrne (2016) presents results from several other studies in a review in Nature. In *D. melanogaster* (fruit fly), a resting period of 15 minutes between LTP-inducing stimulations gave increased LTP response in neurons while a massed protocol of 1 min resting time did not. A study on *A.californica,* (a sea slug species) gave increased LTP response if the interval between inducing pulses were 45 minutes, but not when it was 15 minutes or 60 minutes and a study in rodents saw increased LTP responses at 60 minutes resting intervals, but not at 20 minutes and not at 120 minutes.

The difference in timing in these studies coincides with the syntetization of certain biomolecules. Different ones are active in different species and more than one type is active at the same time in any one species at. One group of such biomolecules is map kinases, (MAPK-family proteins). The synthesis takes some time to be completed and it is speculated that if the second stimulation occurrs before the synthesis time frame, no effect will be observed on the LTP. Likewise, the biomolecules decay over time and if the second stimulation occurrs after too long a decay time, it has been suggested that no effect on LTP would be observed, and none was in any of the reviewed studies.

This new data suggest that spaced learning is at least partially possible to explain by the synthesis of certain biomolecules as a response to stimulation, and also that it should be possible to predict optimal stimulation times by prediction of concentration peaks of the active molecules. An attempt of this has been done in a study by Zhang et al (2012). In the study a differential equation model was used to predict concentration peaks of a few biomolecules active in the sea slug A.Californicum. The concentration peaks were then used to predict an optimal stimulation protocol with irregular intervals between stimulation bursts and the effect was greater than the commonly used stimulation protocols in LTP research on the species.

The irregular spacing was a result from several different biomolecules involved peaking in concentration at different times. This both explains why irregularly spaced protocols are more effective than regular ones when utilizing spaced learning (Bjork, 2016) and also suggest that today's spaced learning schedules could be further improved by predicting human biomolecule concentration peaks for the molecules involved in learning. With the help of computational models, candidates for better learning protocols could then be calculated and tested. This gives hope for great improvements in the future, though much research still remains to be done before we will ever see any of it in the classroom.

Recent research on memory imprinting

It has been hypothesised that memories are stored in clusters of neurons jointly activating when learning or reading the memories. This circuit associated with a memory is referred to as an ***engram***.

The spaced learning explanation is dependent on the assumption that most of the neurons activated during a learning task consist of a single or a few imprints combined, i.e. identical each time. In a study by Takagi et al (2015), a novel method of optogenetics was used. A genetically modified, light sensitive protein involved in controlling dendritic spine growth was introduced in rodents. The protein can be turned on and off using light signals. These signals are much more specific than electric fields previously used, allowing for control very small groups of neurons, instead of general areas of the brain.

This new method's higher precision was used to show that a memory of spcific motor skills is indeed ingrained in small clusters of neurons in different parts of the brain. These parts interconnect with each other and that the variation over time on which neurons are activated when learning and using the motor skill is largely invariant, suggesting that memories do indeed get ingrained in specific neuronal circuits dependent on which neurons are connected to which in a created memory. Turning off the synaptic potentiation terminated the effect, resulting in the rodents forgetting their acquired motor skill and performing no better than a control group. They also did not lose any other acquired motor skills at the same time. This suggests that memories are indeed stored in clusters of neurons activated at the same time during learning and activation of memories. This could partly explain the efficiency of interleaving (Bjork, 2016) when learning a new subject. Even if constantly studying, changing subject allow the clusters of neurons involved in the first memory task to rest while another cluster is activated learning the new subject.

The role of sleep in learning

A recent study by Landmann et al (2015) on the effect of REM sleep on the LTP effect and reorganization of the neural circuits involved in fear suppression in rats suggest that a major part of memory consolidation occurs during REM sleep. REM sleep is the stage of sleeping in which dreaming occurs and is associated with intense neuronal activity resembling that of being fully awake, as compared to the less active stages of the sleep cycle (Myers, 2004). The results of this study lies in line with, though specializing in a very narrow part of the adverse effects from sleep deprivation as generally presented by Division of Sleep Medicine at Harvard Medical School (2007). A recent publication from the Netherlands Association for Sleep Wake Research (2016) further links low school performance and grades with sleep deprivation. This suggest that the, until previously, little studied connection between biochemical mechanisms of learning and mental development during sleep and modern learning theories is a field that could greatly benefit modern education.

Discussion task

This section contains the discussion task prepared as teaching material for a class on modern perspectives of learning. The task was originially written in Swedish and is reprinted in original form. A translation of the task text has been included below.

Caseuppgift för lektion i moderna perspektiv på lärande

Andreas undervisar i matematik på en stor gymnasieskola i södra Sverige och har bland annat en etta i matematik 1C. Klassen har nyligen haft ett delprov under kursen och en elev som det inte gick så bra för har sökt upp honom för att fråga om vad hen kan göra för att klara omprovet. Under samtalet framkommer att eleven upplever att hen försöker jobba hårt under lektionerna, men att det är svårt att koncentrera sig för att hen alltid är så trött, och det känns som om inget fastnar. Nästa lektion är det som gjordes den förra bortglömt. Inför provet har eleven känt sig väldigt oförberedd och panikpluggat innehållet de sista dagarna. Eleven uttrycker förtvivlan över att hen sliter så hårt men ändå inte får resultat för det.

En ganska vanlig situation i en gymnasieklass är att det finns ett flertal elever som av olika anledningar får för lite sömn och ofta är trötta och har svårt att koncentrera sig under skoldagen. Ni har säkert stött på åtminstone ett par sådana elever under er praktik. Många elever har också en dålig studieteknik som ytterligare bidrar till att studierna blir ineffektiva.

Diskutera möjliga sätt för er att hjälpa dessa elever till bättre studieresultat utifrån er roll som lärare för dem. Basera diskussionen både på tidigare erfarenhet och på de texter om moderna perspektiv på lärande som ni har läst inför dagen.

English translation of case for lecture in modern perspectives on learning

Andreas is a teacher in mathematics at a large hicg school in the southern parts of Sweden. One of the classes he is teaching is a freshman class in the course ***matematik 1C***[1]*.The class has recently had the first test of the course and a student who failed the test has contacted him to ask for help to pass a re-test scheduled later on.The student is frustrated, because despite hard work during lectures, the results achieved are low. The student is always tired and has a feeling that nothing learned during classes sticks to the memory. The next class everything done the last one has been forgotten and the student has to start over again. The student felt unprepared for the test, and studied non-stop for it during the last days before it and is expressing feelings of desperation, as all the hard work during classes seems to be for nothing.*

In a high-school class it is common to have several students that, for different reasons, are not getting enough sleep. These students are often tired and have probmlems to focus during the school day. You have probably encountered at least a few of those students during your first internship period. Another common problem is that students have learned no good studying method, which contributes to ineffective learning.

Discuss what you can do to help these students perform better in school from the perspective of teachers of the students. Base it both on previous experience and on the literature on modern perspectives in learning that you read in preparation for today.

[1] Freely translated to mathematics 1C. The course is the first high-school course in matehrmatics for science students in the current Swedish school system.

Some informal reflections of the use of the article and the discussion task

Did the supplied educational article have any impact? Well, since no formal evaluation was done there is no hard data to base any conclusion on. It seems plausible that since the material was more of a supplementary than obligatory nature, it was skipped by some students, though some did read it. Hopefully they did get some new perspectives from it and from the teaching from the lession. The first lessionfocused the first hour on revision of last weeks topics, and the second on the new upcoming material. A few concepts from the "computational learning model" (comparing the brain to a computer, or a computer to the brain) and from neurodidacticals models were discussed. The focus was mainly on the spaced learning and interleaving and the neurological support for those methods was only present as a faint background.

The second lession a week after, was started by the students revising the previous lession by groupwise acting out important aspects in small plays. It became evident that a few more had read the article or at leasr discussed it's contens in preparation of this lesson, as the groups acting did catch many of the core concepts of neurodidactical models presented in the course. The concepts of taking breaks, spaced learning, and interleaving, as well as the importance of sleep was there. After a short break, the students were given the discussion task and I walked around eavesdropping to the "beehives". The students managed to come up with an impressive amount of possible ways to tackle the problem presented, and also did extensive elaboration of the possible causes of the problem, going far beyond what I had expected.

Some suggestions the students came up with, noted down in the order they were brought up follow:

- -More practical work, another type of teaching might activate a sleepy student better
- -Change the subject of the lessions a bit more often, or at least change parts of the subject to get new input.
- "Force" the student to take a break.
- Involve the studenths health team (school nurse, school counselor and other personnel working for the psychococial health of the students at the school) in case there is more behind the student being tired than first meets the eye.
- -Make sure that students really takes a break when there is a break, and don't continue sitting by a computer or cell phone. The body needs fresh air and to move.
- -Create clear connections to earlier lessions, (*to reinforce the engrams formed then, author's note*)
- -Use quizes to get a short break and at the same time reinforce parts of earlier material.
- -Try to increase the motivation of the student inc ase it is low.
- -Mix in other teaching forms, like group activities or other social activites in the teaching
- -Bring it up with the student ,and if a minor, also the parents if needed and talk about possible ways forward. Could there be factors at home causing the lack of sleep?
- -Check with other teachers if it's only the math that is porblematic or if it's a recurring problem.
- -teach proper techniques to study more efficiently. Important to teach several and to empasise that there is no universal method that will fit all and the students need to test to find out if they'll work.

There were also extensive reflections regarding what could be going on behind the scenes (as is reflected in the suggestions for the teacher). Is the cause really that the student is tired all the time or is it a symptom of boredom? Could there be a lack of sleep caused by external factors or is the student's

habit of skipping sleep for the last netflix episode or late night texting the cause? We don't know and it was really impressive how the to-be-teachers sought for solutions without initially blaming the student for the problem, as the text in the case did allow for that interpretatuion. There were discussions of hyposomnia and personal problems as possible reasons and what could be done to help the school situation. Another impressive thing pointed out by the responsible lecturer of the cause when we spoke afterwards was that the discussions focused on what they could do to remedy the situation as teachers to help the student perform instead of on what the student could do.

While this is not strictly related to the neuroscience brought up in the article supplied, it still is strongly connected to the regulation of the reward we have, that can cause positive results but also addiction certain substacnes or behaviours and that definitely affects the work of teachers. What motivates a student? And how do we design envoironments and tasks that trigger the inner motivation for learning? Eavesdropping on the discussions gives me hope for the future. If the “can do”-mentality and eagerness to discuss new problems and use research made available to them of these to be teachers was the norm it might slowly transform the future school and society to embrace research more than has previously been the case.

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