Bachelor's project

NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biomedical Laboratory Science

Siri Margrethe Solberg Lausund & Sabrin Mohamed Said Mahmud

Evaluation and comparison of proliferative markers in detection of neurogenesis in a transgenic rat model of Alzheimer's disease

Bachelor's project in Bachelor i Bioingeniørfag

Supervisor: Atefe R. Tari, Cecilie S. Norevik, Ragnhild E. N. Røsbjørgen, Wenche S. Prestvik

June 2020

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Our heart is in the future

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Writing a bachelor thesis in the midst of one of the biggest pandemics of our time has not been easy, and without the eagerness and knowledge of our supervisors, this would not have been possible. We want to thank PhD candidate Atefe R. Tari, PhD candidate Cecilie S. Norevik and Senior Engineer Ragnhild E. N. Røsbjørgen, not only for their professional guidance and expertise, but for their flexibility and encouragement in a time when things have been anything but normal. We would also like to thank our facilitator Wenche Sletthjell Prestvik for her helpful suggestions and patience throughout the project work.

Even though the thesis unfortunately ended up being a mainly theoretical approach instead of a practical one, we would additionally like to thank CERG for the opportunity to write this thesis and for giving us access to your facilities and laboratory areas.

Oslo & Stockholm, June 3rd, 2020

Siri Margrethe Solberg Lausund

Sabrin Mohamed Said Mahmud

Sammendrag

Antallet mennesker over 60 år har på global basis doblet seg siden 1980. Dette tallet er forventet å øke til rundt 2 milliarder mennesker innen 2050. I Norge er det estimert at omtrent 80 000 – 100 000 mennesker per dags dato lever med en eller annen form for demens. Av de diagnostiserte demenstilfellene står Alzheimers sykdom for cirka 60-80% av disse.

Med økt gjennomsnittlig levealder er behovet for å finne en behandling for nevrodegenerative sykdommer som Alzheimer stor, da alder er en av de fremste risikofaktorene for å utvikle dette. Det er en etablert sannhet at nydannelse av nerveceller, også kalt nevrogenese, forekommer i hippocampus også hos voksne individer. Nevrogenese hos voksne bidrar til å vedlikeholde kognitive ferdigheter knyttet til hippocampus. Nye, og pågående, studier ser på mulighetene for behandling ved hjelp av blodtransfusjon fra unge trente rotter til rotter med Alzheimer sykdom, og hvilken innvirkning dette har på deres nevrogenese.

Ved Cardiac Exercise Research Group (CERG) er det et pågående prosjekt hvor blod fra unge rotter som har fulgt et 6-ukers høy-intensitets treningsprogram, blir overført til rotter fra en rottemodell i et tidlig stadium av Alzheimer Sykdom. Dette gjøres for å undersøke effekten dette kan ha på rotter med Alzheimer sykdom og om det bidrar til økt nevrogenese. Det er kjent fra før at trening øker nevrogenese. Målet med CERG sin prosjektet er å undersøke om injisert trent blod kan ha lignende effekt. Målet for vårt prosjekt var å optimalisere av en immunohistokjemisk metode for påvisning av nydannede nevroner, og sammenligning av dette med andre potensielle prolifereringsmarkører som også kan brukes til dette formålet.

Grunnet utbruddet av SARS-CoV-2 og påfølgende nedstengning av Norge, ble oppgaven vår i hovedsak en teoretisk tilnærming gjort på grunnlag av tidligere forsøk på optimalisering gjort ved CERG, samt ved hjelp av litteratursøk gjort i PudMed, ScienceDirect og Google Scholar. Det vil dermed være nødvendig med videre utprøvning av metodene i denne oppgaven på laboratoriet, for å kunne avgjøre hva som best egner seg til påvisning av nevrogenese hos rotter med Alzheimer sykdom.

Abstract

On a global basis the number of people aged 60 and over has doubled since 1980 and is expected to reach 2 billion in 2050. It is estimated that 80-100,000 people in Norway presently live with some form of dementia. The most frequent diagnosis is Alzheimer's disease (AD), which accounts for 60-80% of the diagnosed cases.

With an increased life expectancy, the need to find treatments for neurodegenerative diseases is imminent, as age is one of the primary risk factors for developing these. An established truth is that neurogenesis occurs in the hippocampus also in adult and aging individuals. This contributes to the maintenance of cognitive skills. New and ongoing studies are looking into the possibility of treating a rat model with developing AD with transfusions of blood from younger, trained rats, and what effects this treatment has on neurogenesis in the model.

At the Cardiac Exercise Research Group (CERG) there is an ongoing project where blood from young rats who are subjected to a six-week high-intensity training program is transfused into AD-model rats in early stages of AD. The model rats are then examined for the effects this has on neurogenesis. It is known that training/physical activity increase neurogenesis. The goal of CERG project is to investigate whether injected trained blood can have similar effect.
The goal of our project was to improve and optimize an immunohistochemical method that may detect newly developed neurons and to compare this proliferation marker with other potential proliferation markers used for this purpose.

Due to the CoViD-19 outbreak and the resultant lock-down in Norway, our thesis has become a mainly theoretical approach on the basis of earlier tests and experiments to optimize detection of neurogenesis at CERG, as well as literature studies using PubMed, ScienceDirect, research gate and Google Scholar. It will be necessary to transfer the methods described here to a laboratory setting in order to make a final conclusion for the best method of detecting neurogenesis in the AD rat model.

Abbreviations

AD – Alzheimer's Disease

APP – amyloid precursor protein

Aβ – beta-amyloid

BrdU – bromodeoxyuridine

CERG – Cardiac Exercise Research Group

DG – dentate gyrus

EdU – 5-ethynyl-2'-deoxyuridine

IF – Immunofluorescence

IHC – Immunohistochemistry

MCI – Mild Cognitive Impairment

NFT – Neurofibrillary tangles

NSPCs – Neural Stem/Progenitor Cells

OB – olfactory bulb

PA – Physical activity

PE – Physical exercise

SVZ – Subventricular zone

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1.0 Introduction

There is an ongoing project by a research group at Cardiac Exercise Research Group (CERG) in which blood from healthy exercised rats is transferred to rats at an early stage of Alzheimer's disease (AD). This is to examine the therapeutic effect exercised blood might have on neurodegenerative diseases by stimulating neurogenesis in the hippocampus. At the start of our bachelor's project, the main objective was originally optimization of a BrdU staining protocol for the detection of neurogenesis in brain tissue, for future use at CERG. Due to the ongoing coronavirus pandemic it was not possible to carry out the planned laboratory work in person, as the facility we were supposed to work in was under lock-down. Because of this we have had to take a theoretical approach to both the methods in question and their protocols.

1. 1 Alzheimer's disease

The global number of people above the age of 60 has doubled since 1980 and is expected to reach about 2 billion in 2050 [1]. In Norway, there are about 80 000 - 100 000 people who have dementia [2]. Dementia is a general term for decline in memory and deterioration of other cognitive skills and is used for several neurocognitive disorders [3]. Dementia is caused by damage in neurons. With neuronal death, patients can develop memory and behavioral issues [4].
Alzheimer´s disease, (AD), is the most common type of dementing diseases, accounting for about 60-80 % of diagnosed cases [5]. It is caused by damage to the neural cells, something that can weaken the patient's ability to perform body activity such as walking [4] .

AD affects people in different ways. The most common, and one of the first, symptoms is gradual loss of the ability to remember and retain new information. The neurons that are involved in forming and storing new information are the first neurons to die. Other mentionable symptoms of AD include confusion in time and place, difficulties in writing and speaking, depression and trouble in understanding visual images and relationships [4]. There is no available diagnostic test for AD. Therefore, the diagnosis of AD in living patients is based on clinical examination (cognitive tests) and the diagnosis is supported by use of clinical biomarkers in cerebrospinal fluid.

AD is characterized by amyloid plaques and neurofibrillary tangles (NFT) consisting of tau protein. Amyloid plaques are large accumulations of beta-amyloid (Aβ) which is a peptide consisting of 36-43 amino acids, that accumulates in the brain. It can disturb the communication between neurons and cause neuronal death [6]. Tau protein helps in binding and stabilization of microtubules in axons which contributes to transport and signal transmission. Abnormally accumulation of tau protein can cause formation of NFTs, which can disturb and block the communication between nerve cells [7].

AD is a devastating brain-disease which can gradually make it harder to express language, cause memory problems and weaken orientation capability. Therefore, it is important to find a treatment against AD. As of now, there is no cure for AD.

1.1.1 Risk factors

The likelihood of developing AD can increase due to risk factors such as age, cognitive impairment, cardiovascular disease, cardiovascular risk factors, social and cognitive engagement, education, and traumatic brain injury. Age is a very important risk factor for AD. People over the age of 65 are more exposed to developing AD, but also people younger than 65 years can develop AD. Those who have more than one first-degree relative with AD have a higher risk of developing the disease. People with mild cognitive impairment (MCI) are also more likely to develop AD. MCI is a condition that causes a mild but measurable change in thinking abilities that are noticeable to the patient and people around him or her, but it does not affect the ability to do everyday activities. Some studies showed that about 10-20 % of people over 65 years of age have MCI. There are about 15 % of those who consulted their doctor about the symptoms they have, have progressed dementia [4]. Several non-genetic risk factors, including recognized vascular risk factors, have been associated with AD [4].

1.2 Effect of physical activity in patients with a high risk of, or with diagnosed, AD

Physical activity (PA) is defined by the World Health Organization (WHO) as “any bodily movement produced by skeletal muscles that requires energy expenditure” [8].

Physical activity should not be used interchangeably with physical exercise (PE), as the latter is a subcategory of PA where the objective is maintenance or improvement of physical fitness, often on a regular schedule. PA is a term covering a range of activities including PE, but also simpler forms of activity such as walking or running, daily chores, working or gardening [8].

WHO recommend that adult individuals aged 65 and above should partake in at least 150 minutes of moderate intensity, or 75 minutes of high intensity, physical activity throughout the week to reduce risk of cognitive decline [9]. Some studies have shown that PA has a beneficial effect on dementia and other MCIs. Two studies demonstrated that regular physical activity was associated with a 30-40 % reduction in the risk of developing AD compared to physical inactive individuals [10,11]. PA may directly protect against dementia through different biological mechanism, including increased hippocampal neurogenesis and neuroplasticity [12].

1.3 Young blood counteracts age-related degeneration

A study in rodents have shown that administration of blood from young mice into old mice counteract age-related degeneration in various tissues, including the brain [13].
Villeda et al. showed that mice receiving young blood displayed greater hippocampal spine density and plasticity than mice receiving old blood transfusions. A pilot study [14] in humans involving young blood transfusion to AD patients, showed that young blood plasma helped AD patients in regaining their ability to perform in everyday activities. The study was conducted over a duration of six months, with patients who had mild to moderate symptoms of AD.
This indicates that there are factors in blood from young individuals, that may have a positive effect on cognitive function and skills when transfused to elderly individuals. In a study done by *Tari et al* , it is hypothesized that exercised blood might have similar properties to young blood in regards to neurogenesis in individuals with neurodegenerative diseases, like AD [15].

1.4 Neurogenesis in the healthy brain

Neurogenesis is the process by which neurons are developed from neural stem cells and is a key factor in embryonic development. It was long believed that the brain and the central nervous system were incapable of neurogenesis and neural regeneration in adults (adult neurogenesis), but it is now well established that neurogenesis occurs throughout life, although at a slower rate [16].

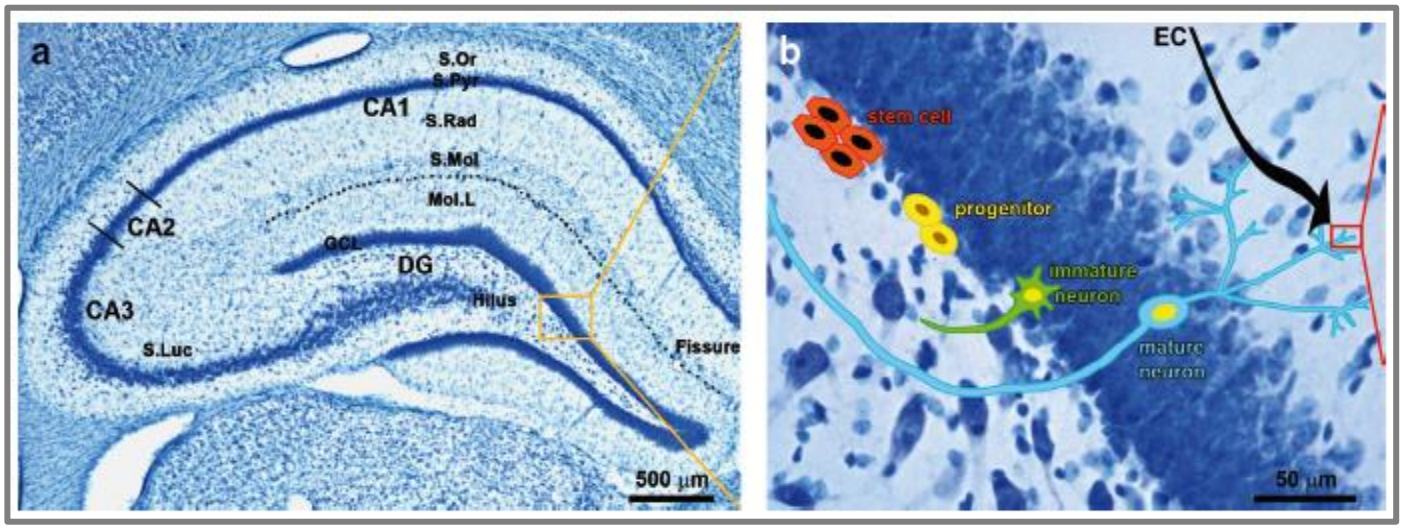

Figure 1: *Neurogenesis in the hippocampus. (a) Anatomical structures of the hippocampus. The dentate gyrus (DG) is highlighted and marked. (b) Schematic illustration of the maturation process of NPSC's in the subgranular zone, into mature neurons. Adapted from Journal of Anatomy, Jose J. Rodriguez, and Alexei Verkhratsky* [17]

Adult neurogenesis has been proven to occur in two main areas of the human brain:
In the subgranular zone, which is part of the dentate gyrus of the hippocampus, and in the subventricular zone (SVZ), where the neurons migrate to the olfactory bulb (OB) and further differentiate and contribute to the sense of smell [18,19]. The hippocampus (shown in figure 1 (a)) is an extension of the temporal cerebral cortex and can be located in the medial temporal lobe. It plays an important role in both memory and spatial navigation [17].

As stated above, the hippocampus is one of two known areas in the brain where adult neurogenesis occurs. Adult neural stem/progenitor cells, hereby referred to as NSPCs, are responsible for neurogenesis in both the subventricular zone, and in the hippocampal dentate gyrus (DG) [19]. NSPCs lay dormant in these areas but undergo proliferation when activated which leads to the formation of new neurons [20]. The newly formed neurons then undergo differentiation

mainly into neuronal cells before they eventually mature and integrate in the surrounding neural tissue, as shown in figure 1 (b). In the dentate gyrus the immature neurons migrate into the granule cell layer where they mature over a period of about three weeks [20].

The knowledge regarding neurogenesis in the adult human brain is limited, as most studies of the neurogenic process is done in rodents and non-humane primates. There is however solid evidence that new neurons are formed in the human brain, and that it is comparable to the amount of neurogenesis in the hippocampal DG in rodents [19].

1.4.1 Synaptic Plasticity

Synapses are the brains main instrument for communication in the neural pathways, as well as for storing information [21]. In short synaptic plasticity is the strengthening or weakening of a synaptic contact which changes as a result of increased or decreased activity of the neural circuits in the brain [22]. As a result of activity or stimuli, the synaptic connections modify to transfer information more efficiently and with greater accuracy. With a lack of activity or stimuli the synaptic pathways weaken and may lead to decreased cognitive function [23]. It is thought that MCI in the early stages of AD might be due to the accumulation of amyloid beta plaques and NFT which leads to synaptic dysfunction and with that reduced plasticity. Even extremely low concentrations are thought to have an adverse effect on the synapses and might lead to negative neural changes earlier than visible symptoms of AD, like neurodegeneration [24].

1.5 Neurodegeneration and neurogenesis in AD

As mentioned earlier, it is known that as age progresses the rate of neurogenesis slows down. Several studies suggest that the hippocampal accumulation of amyloid plaques has a strong impact on NSPCs, and might lead to a reduction in adult hippocampal neurogenesis [17] and thus a decline in structure which in turn leads to hippocampal atrophy and memory dysfunction [25] as shown in figure 2.

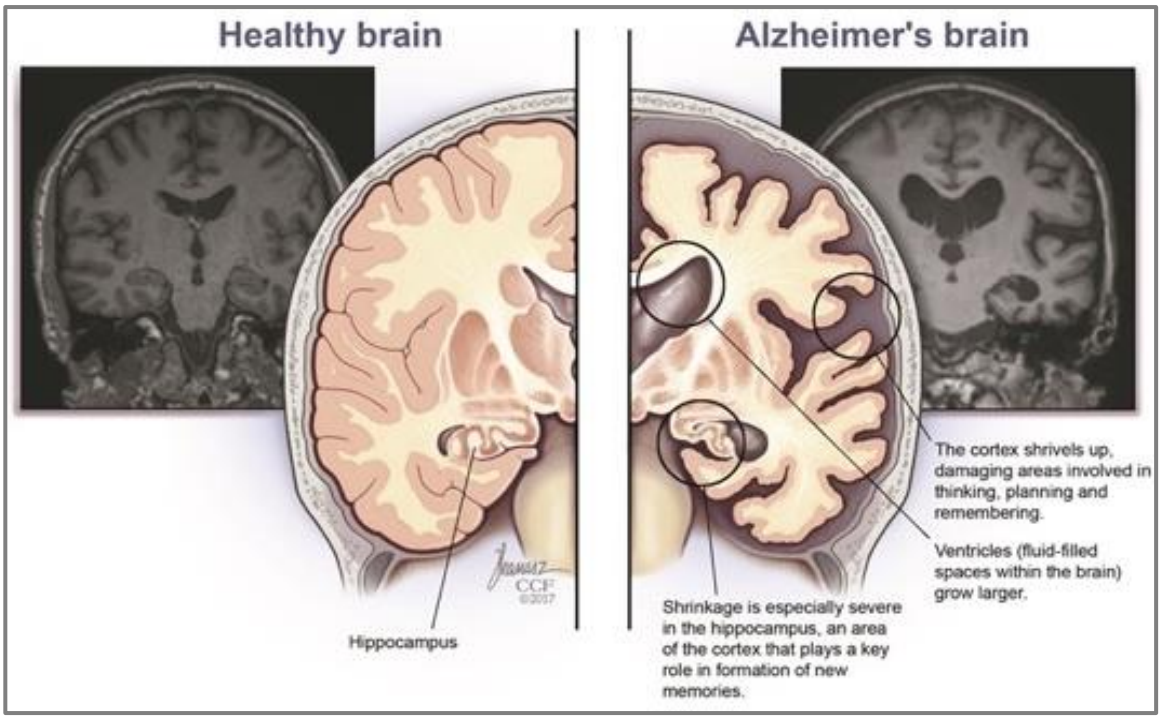

Figure 2: *Differences between a healthy brain, and a brain affected by AD. Adapted from "Keep Memory Alive"*[26].

1.6 McGill-R-Thyl-APP (rat model)

Most cases of AD are sporadic and have an unknown underlying cause. As the cause of sporadic AD is unknown, animal models used in research have been based on the use of animals with genetic mutations associated with familial AD. The reason for this is that animal models with these genetic traits give an almost identical progression of the disease. Either way these genetic models have been invaluable in determining the molecular mechanisms of the progression of AD, and in testing and development of potential therapeutics [27]. As of now there is no single animal model that recreates all aspects of the disease spectrum for AD, but each of the available models allows for a thorough analysis of one or two factors of the disease. This is not possible or ethical with human patients or samples to this date. Rats are preferred over mice, as they are considered physiologically and genetically similar and comparable to humans [28].

The McGill-R-Thy1-APP transgenic rats are among the most studied existing APP transgenic rat lines. Homozygous specimen show age-dependent accumulation of amyloid plaque, cholinergic synapse loss and cognitive impairment [29]. The McGill rat model displays intracellular Aβ

accumulation, visible as early as day seven after birth. At 6-9 months, the primary plaque deposit may be detected within the hippocampus. Additionally, this transgenic model displays cognitive deterioration. Previous studies show that even if there is very little to no Aβ plaque present at three months, several cognitive functions are found altered at this early stage in life [27].

1.7 Immunohistochemistry

Immunohistochemistry (IHC) is a method that can be used in laboratory research and clinical diagnostic. IHC is an important method in laboratory research because of its ability to identify any protein antigen in tissues, which is why it is well suited for use in detection of neurogenesis. The technique used in immunohistochemistry is based on specific antibodies used to find corresponding antigens present in tissue samples, by using antigen-antibody interactions [30]. IHC can be helpful in diagnosing abnormalities in diseases. It is used as a prognostic marker in cancer, in diagnosing tumours of uncertain histogenesis/unknown origin, in predicting therapeutic response in tumours, to identify infection agents, in genetics and to classify neurodegenerative disorders. It can also be used to establish timing of brain trauma [31].

IHC can be used both directly and indirectly. Direct method is the simplest, and the oldest, IHC method. The reaction is a one-step process using a labeled primary antibody [30]. When using a direct IHC method, a labelled primary antibody reacts and attaches to the tissue protein of interest as shown in figure 3. The technique uses one antibody and is fairly quick to complete, but it is rarely used in IHC assays today, as the sensitivity is considered insufficient [32].

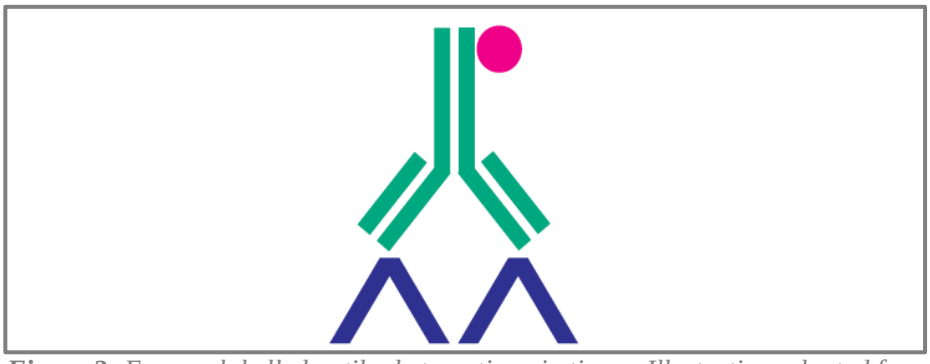

Figure 3: *Enzyme labelled antibody to antigen in tissue. Illustration adapted from Dako Handbook* [32].

In indirect methods, a labeled secondary antibody is used to recognize a primary antibody directed to the protein of interest (figure 4) [33]. The indirect method is more often used, as the method is far more versatile compared to direct methods. A wider array of primary antibodies from the same species can be used with the same conjugated secondary antibody. The indirect method is also preferred because of the high sensitivity since more than one secondary antibody binds to each primary antibody [32]. The primary antibody should not be raised in the same species as that of the sample. This is to avoid possible cross-reactivity in the tissue.

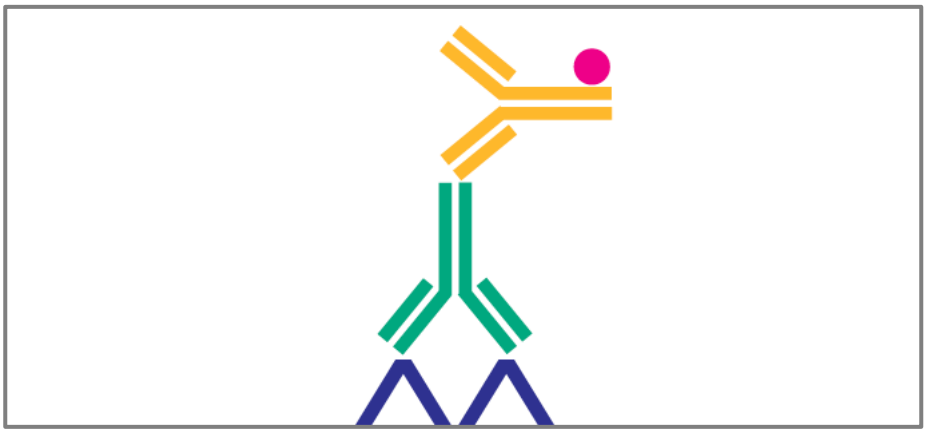

Figure 4: *Indirect method; Enzyme labelled secondary antibody reacts with primary antibody attached to tissue antigen. Illustration adapted from Dako Handbook* [32].

The detection can be visualized by to different ways. The first one is fluorescence detection, where the protein can be detected by using antibody conjugated to fluorescent dyes to evaluate cells [34]. The other one is chromogenic detection, that detects antigen by using an enzyme, which converts soluble substrates into insoluble colored product.

1.7.1 Fluorescence detection

Fluorescence detection or immunofluorescence is an antigen-detection test used mainly on frozen tissues, cells or cultured cells [34]. The method uses a fluorochrome, that emits light in a specific wavelength. The fluorochrome can either be conjugated directly to the primary or the secondary antibody [35].

There are two types of immunofluorescence: Direct and indirect immunofluorescence. The direct method only uses a primary antibody, while the indirect method uses both primary antibody and secondary antibody. A special microscope is used to detect the emitted light at a specific wavelength.

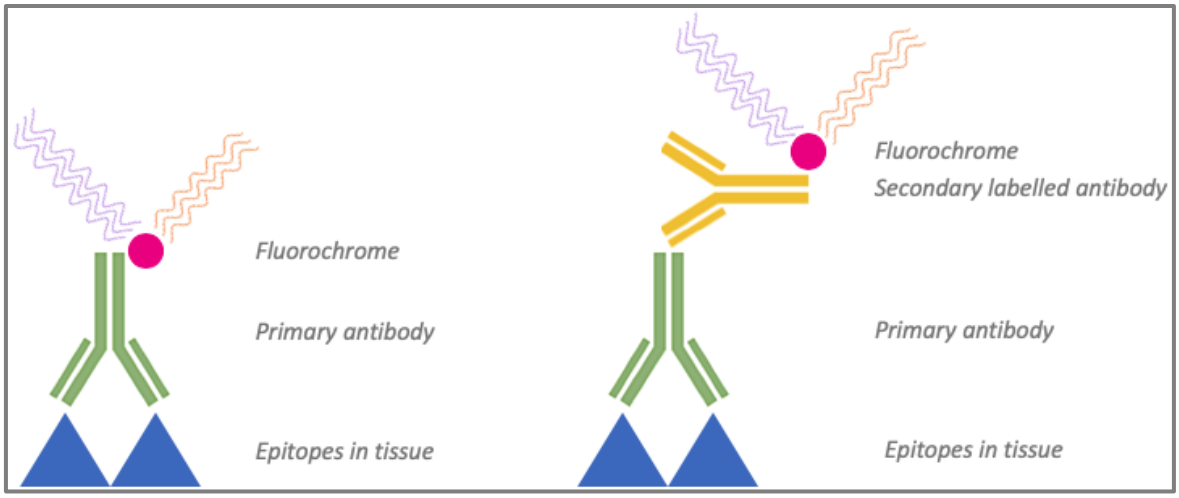

Figure 5: *Illustration showing the principles of direct and indirect staining methods. UV-light alters the state of the fluorochrome which leads to it emitting visible light. Adapted from the Norwegian Lexicon* [36].

1.7.2 Chromogenic detection

Chromogenic detection is an IHC method that uses enzymes to convert soluble substrates to insoluble, chromogenic products [37]. Chromogenic substrates are added to the tissues, which are incubated with an enzyme-conjugated antibody. The most used enzyme is Horseradish peroxidase (HRP), and it can be used with many other chromogenic substrates such as DAP and hydrogen peroxide. DAP oxidized by hydrogen peroxide, and HRP catalysis the reaction. The oxidized DAP makes a brown precipitate. The precipitate that can be used with and make the substrates change color to brown. Hydrogen peroxide must be available, so the DAP can be oxidized and form colored precipitate. The chromogenic precipitate can be seen in tissue by using a normal light microscope, which contributes to making the method is easy to use[38].

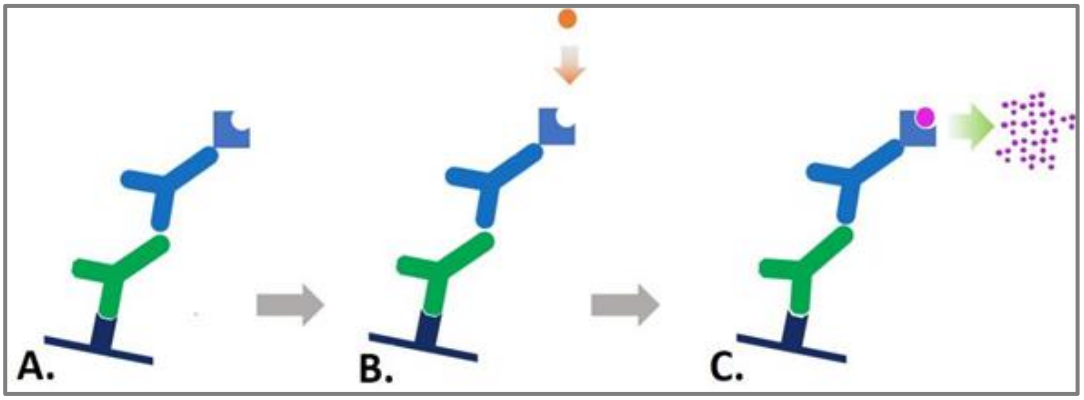

Figure 1: *Indirect colorimetric detection. A. a reporter enzyme-conjugated secondary antibody detects primary antibody for the wanted protein. B. Chromogenic substrate added to the antibody. Antigen complex. C. Reporter enzyme catalyses the modification of the chromogenic substrate to a coloured precipitate. Adapted from Jackson Immuno Researrch* [38].

1.7.3 Control material

When using an IHC method, the use of proper control material is essential to be able to confirm valid staining results. Negative tissue controls should be fixed and embedded identically to the unknown sample tissue but should not contain the targeted tissue marker [32]. The same criteria are applicable for the positive control material, but it should contain the targeted tissue marker or protein. To monitor loss of sensitivity it could be beneficial to utilize a control that is marginally positive, as it allows for the degree of differentiation achieved to be observed [32].

1.8 BrdU

5'bromo-2'-deoxyuridine (BrdU) is a popular cell tracer used to detect and measure proliferating cells in living tissue (in vivo). By substituting thymidine during DNA synthesis in the cell cycle, BrdU is incorporated and passed on to the daughter cells after replication.

Because of the ability to substitute thymidine in the S-phase, it is possible to use BrdU as an exogenous cell tracer in combination with endogenous neuronal markers to assess neurogenesis within the adult central nervous system [39]. By using specific primary antibodies that match these markers, it is possible to visualize changes or development in the neural tissue (neurogenesis) by using secondary antibodies labelled with a fluorescent tag [39].

BrdU can both be injected intraperitoneally or locally in the brain but can also be administered through drinking water (peroral administration). The experimental design determines the dose and number of injections. The dosage ranges from 50 to 300mg per kilogram of body mass in rats. With intraperitoneal BrdU injections the dosage is also determined in relation to the experimental time frame. Higher doses, up to 300mg/kg, are used when the time frame of the experiment is short, and the age of the cells are of importance [39].

1.9 Ki67

As BrdU may have some issues that can affect the staining result, other methods may be used instead of, or in combination with, BrdU. One of the most used markers is Ki67, which is a protein that is associated with cell proliferation and can be utilized as an endogenous marker.

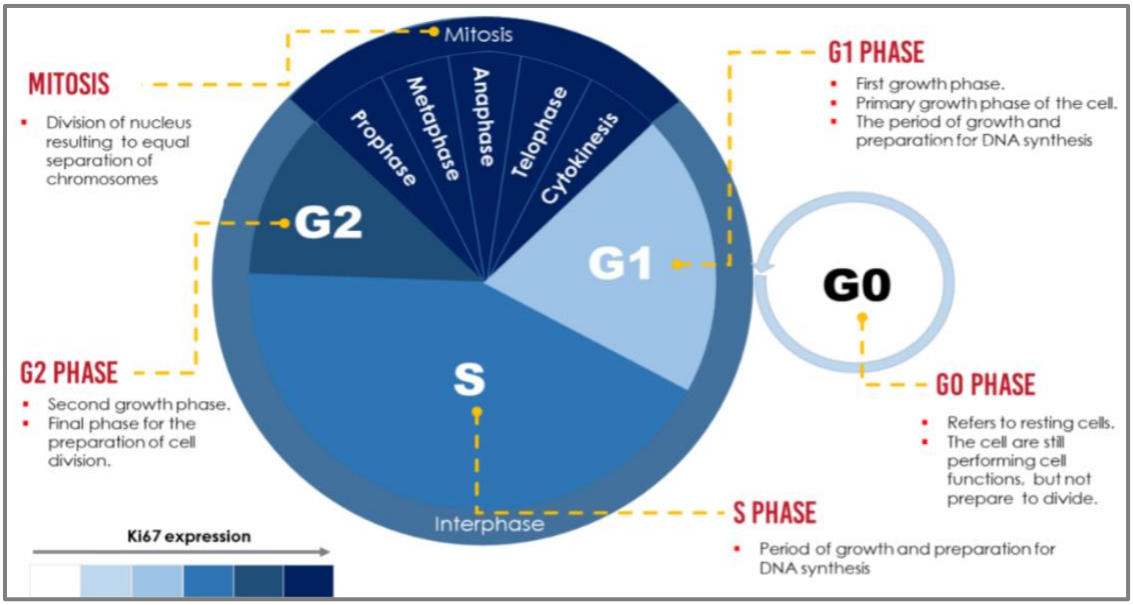

Figure 7: *Ki-67 expression during cell cycle. Adapted from "An Overview of recent counting methods for Ki67 IHC staining"* [40].

The true function of Ki67 is not yet known, but it is considered a reliable marker and is much used to detect cell proliferation. This is because Ki67 is expressed in almost all phases of the cell cycle except at the G0-level (figure 7). It is however expressed at lower levels in the early stages of the G1-phase compared to the S-, G2 and M-phase. This is because the protein mainly starts to become active at the end of the G1-phase and stops when the daughter cell exits the

cycle [39]. Some studies have found that Ki67 is expressed during mitosis in all mammalian species, which includes rodents [41].

1.10 EdU

5-ethynyl-2′-deoxyuridine (EdU) is a thymidine analog that incorporates into newly synthesized DNA. It is a new method and as with BrdU it labels proliferating cells by incorporation in the cell cycle. EdU assays are not dependent on denaturation of DNA or unmasking of sample tissue, to be detected. As shown in figure 8, it uses an alkyne-handle which allows for binding with an azide-containing fluorescent probe which utilizes a highly efficient click chemistry reaction to bind in the sample tissue. EdU preserves the physical integrity of the tissue since DNA denaturation is not necessary, which allows additional protein markers to be used if needed or wanted. A study done by *Zeng et al.* prove that EdU staining can be used as an alternative to BrdU as a proliferative marker in adult neurogenesis [42].

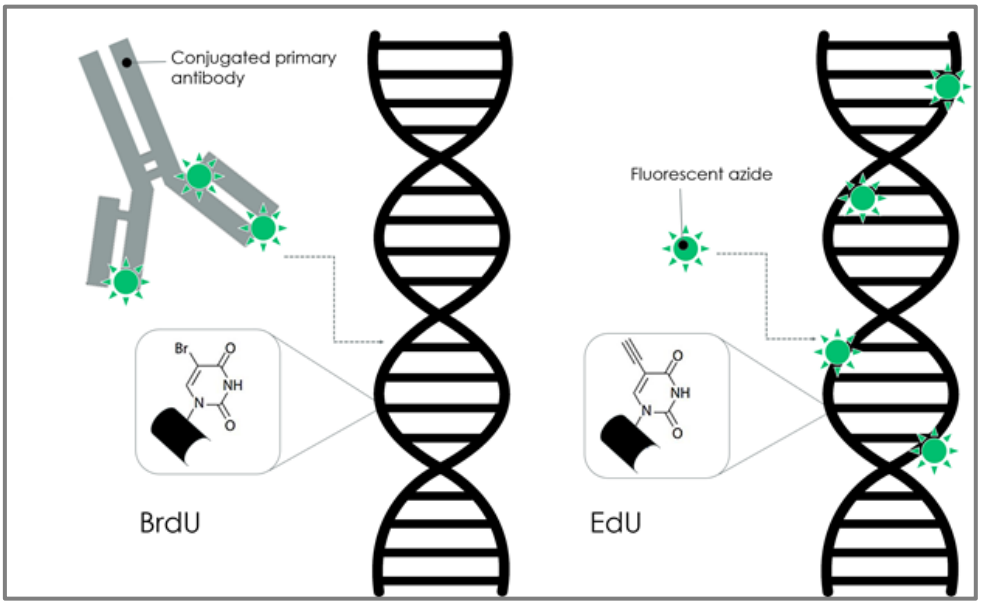

Figure 8: *BrdU (left) requires the DNA to be fully denatured in order to bind to an anti-BrdU primary antibody. EdU (right) relies on 'click' chemistry, where a fluorescent azide can freely bind the EdU molecule. Graphic illustration adapted from Abcam [43].*

1.11 Learning outcome

The purpose of this thesis is to evaluate and compare proliferative markers for detection of neurogenesis in an AD rat model.

How can the current BrdU protocol be modified to improve IHC staining and what other proliferative markers might be relevant in the detection of neurogenesis in rats with Alzheimer's diseases?

2.0 Materials and methods

This thesis was done in theory due to the SARS-CoV-2 outbreak. It ended up being a theoretical approach to a quantitative method of research.
We based our thesis on previous optimization trials done at CERG, as well as literature searches done mainly in PubMed. The search words used are "adult neurogenesis", "hippocampal neurogenesis", “Alzheimer’s Disease”, “neurogenesis in Alzheimer’s disease “"BrdU", "EdU" and "Ki67". The rats used in the previous trials were provided by the laboratory of Professor Menno Witter at the Kavli Institute for Systems Neuroscience and Centre for Neural Computation at NTNU in Trondheim.

2.1 Injection of rats

The following paragraphs have been written under guidance from our supervisors, and thus have no direct origin or source.

The donor rats (used in the ongoing research at CERG) were separated into two groups: One exercised group (marked in green in figure 9) where the rats have been subjected to a six-week high-intensity exercise program, and one sedentary group (marked in yellow in figure 9) who functions as a control group. After the six-week period, the blood from the donor rats was collected before it was centrifuged, aliquoted and stored in a freezer at -80ºC. This ensured the ability to inject several batches of AD rats at different times.

The injection process of the AD rats was done in two parts: The first part consisted of intraperitoneal injections of BrdU or saline solution, and the second part was injection of plasma from either the exercised or sedentary donor rat groups, administered in the tail vein of McGill-R-Thy1-App rats (AD-rats), all while sedated. In addition, a third group was injected with a saline solution instead of plasma (marked in gray in figure 9) and functions as a form of double control group. The injections were administered at a three-day interval before sacrifice after ten injections. The dosage administered was calculated based on the individual weight of the rats. At

CERG the injections were done to between 5 to 7 rats at a time, as it eases the logistics of the tests done prior to sacrifice. The rats were tested in several parameters as shown in figure 9.

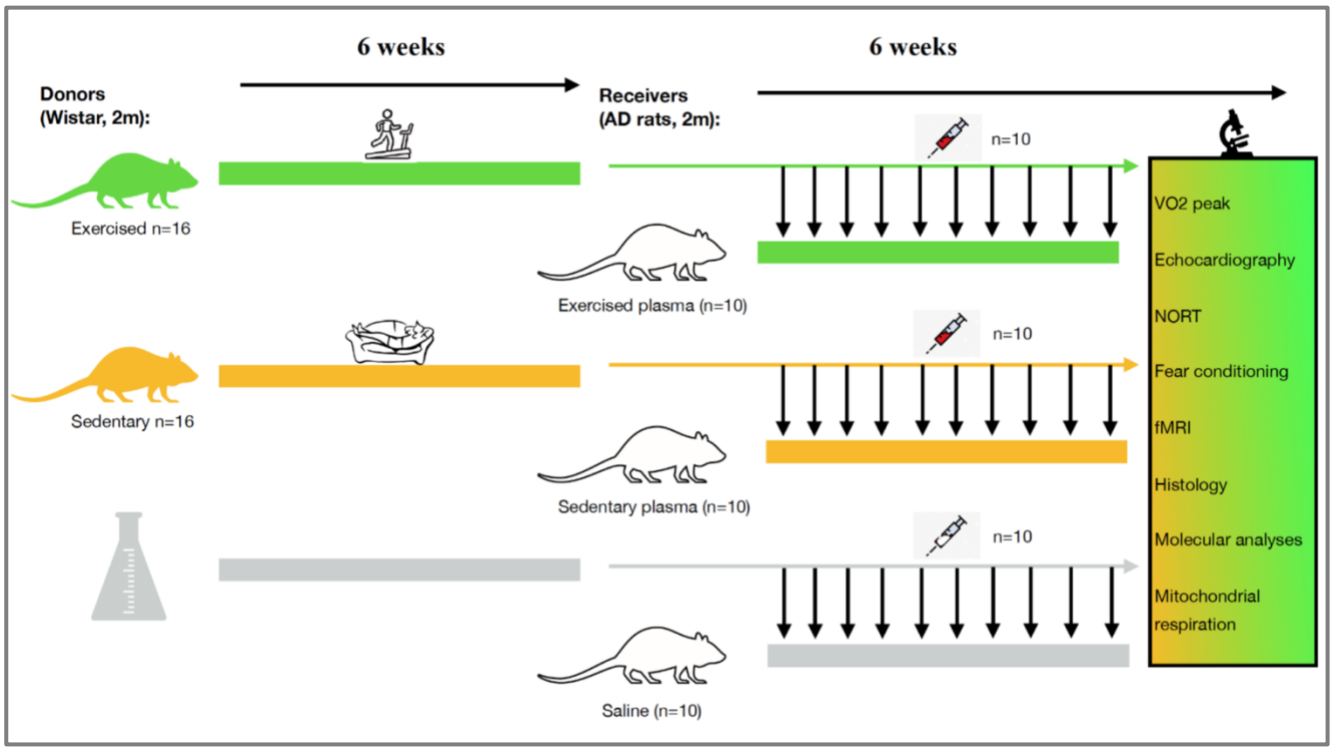

Figure 9: *Illustration showing the activity of the donor rats up until collection of the exercised or sedated blood, followed by the injection process of the AD-rats. As the donor-blood is stored in a freezer the duration between collection of donor-blood and the injection of AD-rats is flexible. (Source: CERG)*

2.2 Preparation and sectioning of tissue

The animals were perfused (by transcardial perfusion) to preserve the brain tissue. They were first anesthetized by using 5% isoflurane, then injected with an overdose of pentobarbital. Before any further progress, the pain reflexes of each animal were checked.

The animals were then opened right below the diaphragm to expose the heart.
The right atrium was cut, and a needle was injected into the apex of the left ventricle. A peristaltic pump (Thermo Fisher Scientific, Massachusetts, USA) was used to pump Ringer's solution (145mM NaCl, 3,35 mM KCl, 2,28 mM NaHCO3) to replace the blood, followed by 4% paraformaldehyde (PFA) to fix the body.

Lastly the brains were dissected and placed in 4% PFA for 1-2 days, and then stored in a DMSO solution (2% dimethyl sulfoxide in 125mM PB and 20% glycerol) at refrigerator temperature. For

slicing of the tissue, a microtome (Microm HM430, Thermo Fisher Scientific) was used to cut the brains into 40 micrometer thin sections in five series where four were placed in tubes containing DMSO solution. The remaining slice was mounted on a super-frost glass slide. The slices stored in tubes are the ones used for immunostaining.

2.3 BrdU assay protocol

The entire protocol is shown in Appendix II.
The tissue samples were rinsed in Tris Buffered saline* (TBS) and incubated in 1M HCl at 45°C for 30 minutes, before going through another rinse in TBS followed by 2 hours of unmasking in 0.125 M phosphate buffer (PB) at 60°C. After another two rinsing steps in PB and TBS with 0.5% Triton X-100 (TBS-Tx), the sections were incubated with 10% Goat serum for 1 hour to block nonspecific binding from the secondary antibody. The samples were then incubated with anti-BrdU Primary antibody (1:10) in a refrigerator overnight.
The following day the sections were rinsed in TBS-Tx to remove excess antibody from the tissue, and then incubated with Goat Anti-Mouse IgG H&L Alexa Flour 488 secondary antibody (1:100) for 2 hours at room temperature. Subsequently the tissue was then rinsed in TBS, mounted on glass slides and coverslipped with Vectashield.

2.3.1 Injection of BrdU

With intraperitoneal BrdU injection the dose is also determined in relation to the experimental time. Higher doses, up to 300mg/1kg, are used when the time frame of the experiment is short, and the age of the cells are of importance [39]

The BrdU used for injection at CERG was provided by Abcam (BrdU Thymidine analog (ab142567)) [44].

All rats were injected with BrdU or NaCl in parallel with plasma transfusion (or injected with saline solution if the rat was in the control group), all while sedated.

2.3.2 Antibodies and control material

Table 1: List of antibodies for immunostaining [45]

Antibody		Recommended dilution from the supplier	Clonality	Supplier
Primary antibody BrdU	Anti-BrdU antibody (mouse) ab8152	1:5 – 1:10	Monoclonal	Abcam
Secondary antibody	Goat Anti-Mouse lgG H&L (Alexa Flour 488) ab150113	1:200 (1:100 used)	Polyclonal	Abcam
Primary antibody (to be further tested)	BrdU Antibody (mouse) B35128	Assay dependent*	Monoclonal	ThermoFisher SCIENTIFIC

Control material for immunostaining:

BrdU control slides (IHC) (ab129956) [46].

2.4 Protocols for other potential proliferative markers

2.4.1 Ki67 assay protocol

To analyze tissue with Ki67, the tissue must be washed with PBS to remove sodium azide. Further it has to be incubated with a Ki67 antibody for 48 hours at 48°C. The tissue subsequently has to be rinsed three times with PBS for 5 minutes, to remove excess unbound antibodies. It is then incubated for two hours with a fluorescent conjugated secondary antibody that attaches to the matching and available epitopes in the sample tissue. After the two-hour incubation, the tissue is rinsed once more in PBS to remove excess secondary antibody, before it is mounted on a glass slide to prepare it for microscopy [47].

2.4.2 EdU assay protocol

EdU staining is based on a Click-iT™ imaging kit provided by several manufacturers. It is mainly intended to be used in cell cultures but has been shown to be adaptable to use in IHC. The following protocol is based on a protocol originally adapted by *Zeng et al* [42].

The tissue samples are washed twice with 3% bovine serum albumin (BSA) in PBS to block unspecific binding, and then permeabilized with TBS-Tx for 20 minutes. Further the tissue should be washed twice again with 3% BSA in PBS to remove excess solution. Subsequently the tissue is incubated for 30 minutes in a Click-iT™ solution that contains Click-iT™ reaction buffer, CuSO4, Alexa Fluor® 594 Azide, and a reaction buffer additive, while it is protected from any light. After this the tissue is washed once more in 3% BSA in PBS. If wanted, the tissue can be washed once with PBS and be further incubated with a DNA stain. The whole procedure can be carried out at room temperature.

3.0 Results and discussion

3.1 Evaluation of the BrdU protocol

The purpose of this thesis is to identify possible procedural and systematic changes that will optimize the staining procedure in brain tissue of AD rats. In previous trials at CERG using BrdU, they have not been able to achieve adequate differentiation and the staining procedure has not met the expected results. As shown in figure 10 (a) and (b) it can be difficult to separate the staining attempts if the sample origin is unknown. In figure 10 (b) the hippocampus of a BrdU-injected rat is shown. A clearer distinction is expected between the brains injected with BrdU and those injected with only a saline solution. This is due to avaliable, or in the saline injected rat lack of avaliable, BrdU epitopes in the tissue.

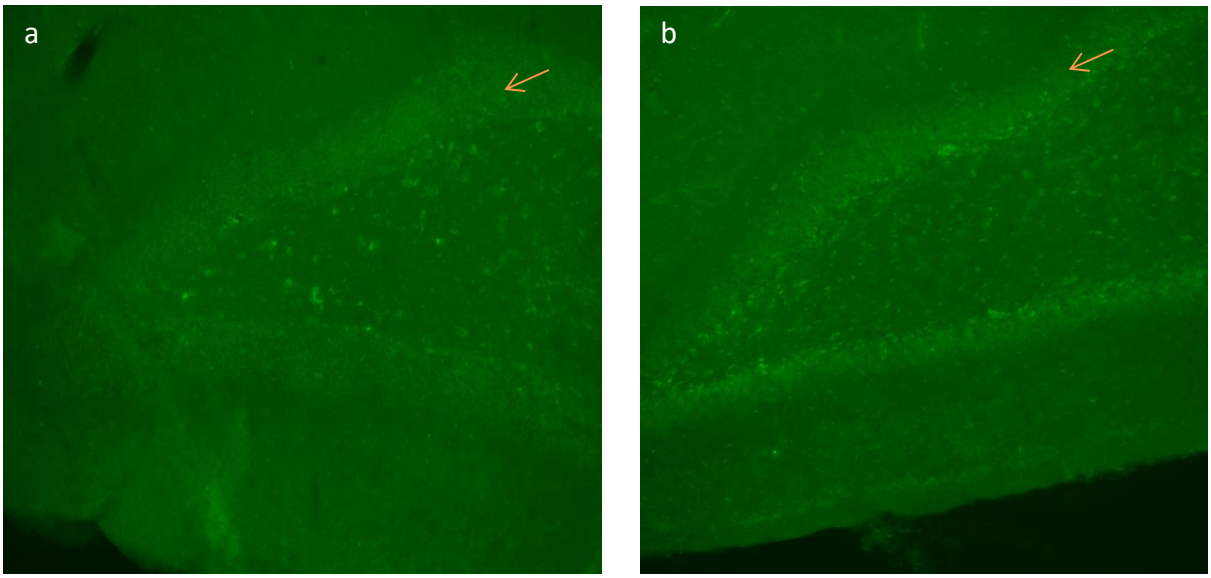

Figure 10: *(a) Brain section with BrdU-staining of AD rat injected with just a saline solution. In this stain it is not expected any positive results. (b) Brain section with BrdU staining of AD rat injected only with BrdU. In this stain it is expected to see positive staining in the DG (shown with an arrow). Not an optimal stain. (Source: CERG).*

When brain tissue is stained with BrdU to detect neurogenesis, it is expected that the positive staining will take place in the granule cell layer of the DG (marked with arrows in both figure 10 (a) and (b)). In an optimal stain a much clearer differentiation it is expected in the tissue both with and without BrdU. In cases where this is hard to achieve it can be beneficial to perform a double stain with another antibody like anti-NeuN or anti-Prox1 that stain all neurons, not just the

newly synthesized ones. Tissue that is double stained with BrdU and anti-NeuN can be seen in figure 12. The additional stain is helpful to make sure that the BrdU is incorporated in the new neurons, and that it is not positive in other cell structures like glial cells. It is also helpful to eliminate background stain.

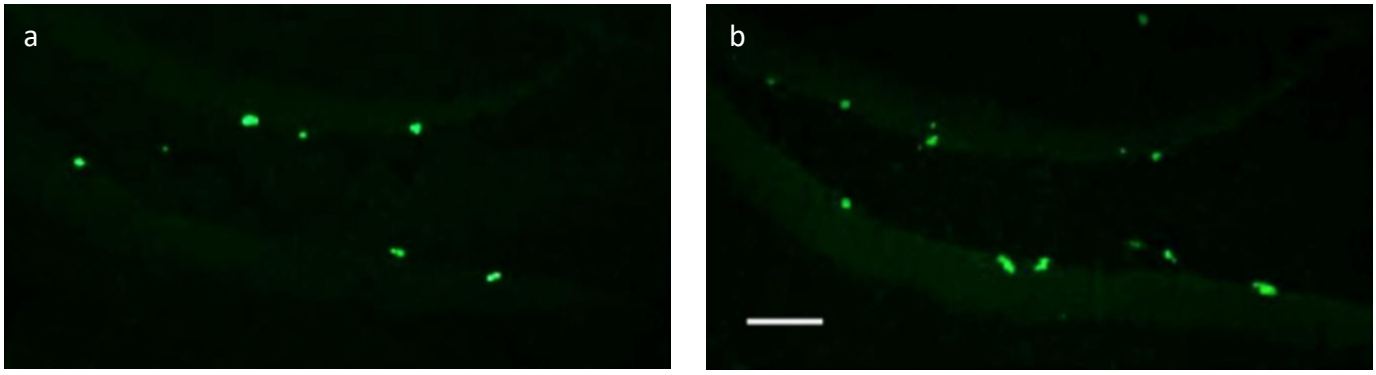

Figure 11: *(a) Expected result for control tissue stained with BrdU. (b) Expected positive result in sample stained with BrdU. Adopted from Zeng et al* [42].

BrdU has some disadvantages that may affect the result. The BrdU may not reach the brain tissue under the injection because of for example blood brain barrier, which can prevent BrdU from penetrating the tissue. The handling and injection procedure can have an unintended effect on neurogenesis as it may inhibit neurogenesis. This might not be the case at CERG as the rats are sedated whilst receiving both blood transfusion and BrdU or saline injections.
Tissue fixation can also affect the result if the tissue is over or under fixated. Over fixation of the tissue, and with that the antigen, can lead to unavailability of antigen epitopes and therefore decrease the staining. In the other way under fixation may not preserve the antigen comprising detection. To fix that it will be needed to shorten or lengthen fixation time [48].

When administered through intraperitoneal injection the incorporation time for BrdU to cells that have a high rate of proliferation may be detectable in as little as 30 minutes after injection. Other tissues that have a lower proliferation rate might need around 24 hours to fully incorporate. Because of the variations in proliferation and incorporation time for the different tissues, it is important to schedule time of injection and dosage needed to match the tissue of interest.

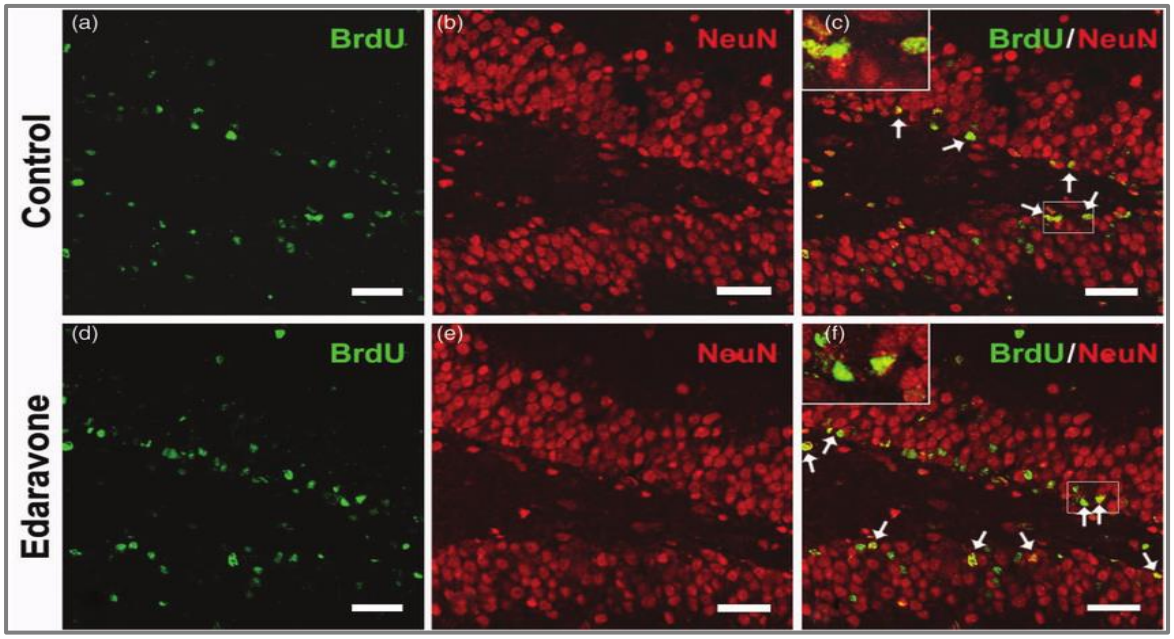

Figure 12: *Tissue that is double stained with BrdU and anti-NeuN. Adapted from "Pre-and Posttreatment. With Edaravone Protects CA1 Hippocampus and Enhance Neurogenesis in the Subgranular Zone of Dentate Gyrus After Transient Global Cerebral Ischemia in Rats"* [49].

3.1.1 Storage

The BrdU solution should be stored at 4ºC for a maximum of one week, or at -20ºC if it is needs to stored for longer period of time. The BrdU solution is especially sensitive to light and should be stored in a dark place to avoid photobleacing.

3.1.2 Antibodies

The antibodies used in pervious stains at CERG were chosen based on recommendations from other collaborators. The first attempts were done with an antibody supplied by Abcam. The antibody went through thorough testing only to reveal that it might be defect. A new batch of antibody was delivered to CERG by Abcam. This was not optimized for staining the sample material at hand. It could be beneficial to run a trial on an antibody supplied by Thermo Fischer to see if any improvement could be detected. Recommended dilutions can be found in table 1.

3.1.3 Hypothetical sources of error

A source of error to consider when hypothesizing sources of error prior to the BrdU staining is the administration of injections. There is no guarantee that the injection procedure is correctly performed, which might lead to poorer incorporation of BrdU in the targeted tissue based on the amount of BrdU actually injected in the rats. The injection process itself might also be a source of error in relation to the detection of neurogenesis in the rats, as repeated injections could be perceived as a stressful experience to the animals. Stress significantly reduces the rate of neurogenesis in the DG. As the rats used at CERG were sedated when receiving the injections, it is possible that the process did not cause the rats to experience any significant amounts of stress.

3.2 Comparison of different proliferative markers

Table 2: Comparison of the selected possible proliferative markers

	BrdU	**EdU**	**Ki67**
	Exogen	Exogen	Endogen
Selectivity	S-phase	S-phase	Detectable in all phases except G0
Administration	Intraperitoneal injection	Intraperitoneal injection	No injection needed
Incorporation in tissue	Denaturation necessary	Denaturation not necessary	Denaturation not necessary
Side effect	toxic	Not significantly toxic	Not toxic

3.2.1 Ki67 - Differences from BrdU

BrdU and Ki67 differ in several ways. One notable difference between Ki67 and BrdU is that Ki67 is an endogenous marker and does not affect living cells in a negative way. Since BrdU is an exogenous marker, it might have adverse effects on living cells [47]. Another difference is that BrdU is only expressed in S-phase, while Ki67 is detectable in all phases of cell cycle except G0-phase. By being detectable in almost all phases, Ki67 might not be suited for age determination of the neural cells. The incorporation of BrdU depends on the dose and the diffusion barrier, but Ki67 does not depend on them [47]. A disadvantage of using Ki67 is that the epitopes to which the antibodies are directed may be masked due to the fixation process. A heat retrieval step is therefore required to allow binding of Ki67.

3.2.2 EdU - Differences from BrdU

The difference between BrdU and EdU is that BrdU-antibodies can only react with single stranded DNA. This is because double stranded DNA blocks anti-BrdU antibody access to incorporate BrdU- molecules. This can expose samples to tough denaturation conditions, which can increase the analysis duration. EdU on the other hand has a streamlined detection protocol, that can reduce the steps and the duration of the analysis [50].

EdU is also not antibody based and does not depend on DNA denaturation of the incorporated nucleosides to bind in tissue [51].

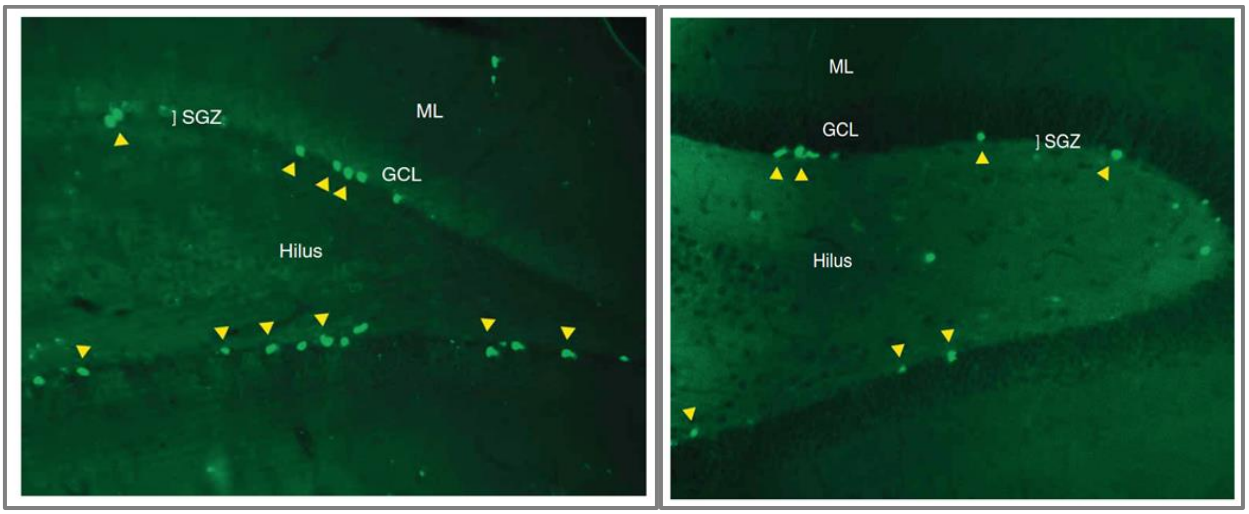

Figure 13: *(a) Coronal section of the DG of a rat stained with BrdU, with a positive result. The Injections was given intraperitoneally at 4h intervals.The yellow arrows show newly formed neural cells in the SGZ. (b) Coronal section of the DG of a rat stained with Ki67, with a positive result. The yellow arrows mark newly formed neural cells in the SGZ. Source: "BrdU assay for neurogenesis in rodents"* [39].

4.0 Conclusion

Previous to this thesis, the current BrdU protocol has been thoroughly tested with varying degrees of staining results, most of which were negative. It may be beneficial to combine BrdU with Neu-N to confirm that BrdU is incorporated in the neural cells. By utilizing an additional tissue marker, it can also be easier to distinguish whether there is poor staining or if there is a negative result. It might also be beneficial to test a new antibody from another supplier, as originally intended in this thesis, as the match of the used antibodies might not be optimal based on variations in quality and sensitivity. A mismatch in antibody to antigen of interest will result in a negative stain.

A promising new proliferative marker besides BrdU is the EdU assay. The protocol is less time consuming and requires fewer steps than BrdU. By not having to denature DNA the integrity of the tissue is also preserved, which makes it easier to combine with additional protein markers. It is also possible to co-label proliferating neural cells using both methods. With using a new method for detection of neurogenesis, a positive control provided by the supplier is essential to confirm or affirm positive staining results. It might also be beneficial to have a negative control provided by the supplier, if this is possible to obtain.

Lastly, the protocols discussed in this thesis needs to be tested in a laboratory for an accurate and fair comparison.

5.0 References

1. WPA2017_Highlights.pdf. Accessed June 1, 2020. https://www.un.org/en/development/desa/population/publications/pdf/ageing/WPA2017_Highlights.pdf

2. Demens. Folkehelseinstituttet. Accessed May 13, 2020. https://www.fhi.no/nettpub/hin/ikke-smittsomme/demens/

3. Dementia. Accessed May 13, 2020. https://www.who.int/news-room/fact-sheets/detail/dementia

4. 2014 Alzheimer's disease facts and figures. *Alzheimers Dement*. 2014;10(2):e47-e92. doi:10.1016/j.jalz.2014.02.001

5. facts-and-figures-2018-r.pdf. Accessed June 1, 2020. https://www.alz.org/media/documents/facts-and-figures-2018-r.pdf

6. Alzheimer's Assosiation Beta-amyloid and the amyloid hypothesis. Published online 2017:4.

7. What Happens to the Brain in Alzheimer's Disease? National Institute on Aging. Accessed June 1, 2020. https://www.nia.nih.gov/health/what-happens-brain-alzheimers-disease

8. Physical activity. Accessed June 2, 2020. https://www.who.int/news-room/fact-sheets/detail/physical-activity

9. WHO | Physical Activity. WHO. Accessed May 13, 2020. http://www.who.int/dietphysicalactivity/pa/en/

10. Aarsland D, Sardahaee FS, Anderssen S, Ballard C, the Alzheimer's Society Systematic. Is physical activity a potential preventive factor for vascular dementia? A systematic review. *Aging Ment Health*. 2010;14(4):386-395. doi:10.1080/13607860903586136

11. prevent.pdf. Accessed May 22, 2020. https://www.choiceforum.org/docs/prevent.pdf

12. van Praag H. Neurogenesis and Exercise: Past and Future Directions. *NeuroMolecular Med*. 2008;10(2):128-140. doi:10.1007/s12017-008-8028-z

13. Villeda SA, Plambeck KE, Middeldorp J, et al. Young blood reverses age-related impairments in cognitive function and synaptic plasticity in mice. *Nat Med*. 2014;20(6):659-663. doi:10.1038/nm.3569

14. Young blood | Stanford Medicine. Accessed May 13, 2020. https://stanmed.stanford.edu/2018winter/clinical-trial-finds-blood-plasma-from-young-donors-promising-for-Alzheimers.html

15. Gontier G, Iyer M, Shea JM, et al. Tet2 Rescues Age-Related Regenerative Decline and Enhances Cognitive Function in the Adult Mouse Brain. *Cell Rep*. 2018;22(8):1974-1981. doi:10.1016/j.celrep.2018.02.001

16. What is neurogenesis? Published November 22, 2016. Accessed May 23, 2020. https://qbi.uq.edu.au/brain-basics/brain-physiology/what-neurogenesis

17. Rodríguez JJ, Verkhratsky A. Neurogenesis in Alzheimer's disease. *J Anat*. 2011;219(1):78-89. doi:10.1111/j.1469-7580.2011.01343.x

18. Olfactory bulb | anatomy | Britannica. Accessed May 13, 2020. https://www.britannica.com/science/olfactory-bulb

19. Braun SMG, Jessberger S. Adult neurogenesis: mechanisms and functional significance. *Development*. 2014;141(10):1983-1986. doi:10.1242/dev.104596

20. Lazarov O, Mattson MP, Peterson DA, Pimplikar SW, van Praag H. When Neurogenesis Encounters Aging and Disease. *Trends Neurosci*. 2010;33(12):569-579. doi:10.1016/j.tins.2010.09.003

21. Mekanismer for hukommelse. Universitetet i Bergen. Accessed June 1, 2020. https://www.uib.no/nb/biomedisin/68159/mekanismer-hukommelse

22. Heinbockel T. Introductory Chapter: Mechanisms and Function of Synaptic Plasticity. *Synaptic Plast*. Published online June 21, 2017. doi:10.5772/67891

23. Jansen J. plastisitet. In: *Store medisinske leksikon*. ; 2020. Accessed June 1, 2020. http://sml.snl.no/plastisitet

24. Skaper SD, Facci L, Zusso M, Giusti P. Synaptic Plasticity, Dementia and Alzheimer Disease. *CNS Neurol Disord Drug Targets*. 2017;16(3):220-233. doi:10.2174/1871527316666170113120853

25. Hippocampal Atrophy and Memory Dysfunction Associated With Physical Inactivity in Community-Dwelling Elderly Subjects: The Sefuri Study - PubMed. Accessed June 2, 2020. https://pubmed.ncbi.nlm.nih.gov/28239530/

26. Alzheimer's Brain | Keep Memory Alive. Accessed May 13, 2020. https://www.keepmemoryalive.org/brain-science/alzheimers-brain/

27. LaFerla FM, Green KN. Animal Models of Alzheimer Disease. *Cold Spring Harb Perspect Med*. 2012;2(11):a006320-a006320. doi:10.1101/cshperspect.a006320

28. A Novel Transgenic Rat Model With a Full Alzheimer's-like Amyloid Pathology Displays Pre-Plaque Intracellular Amyloid-Beta-Associated Cognitive Impairment - PubMed. Accessed June 1, 2020. https://pubmed.ncbi.nlm.nih.gov/20164597/

29. McGill-R-Thy1-APP | ALZFORUM. Accessed May 13, 2020. https://www.alzforum.org/research-models/mcgill-r-thy1-app

30. Chen X, Cho D-B, Yang P-C. Double staining immunohistochemistry. *North Am J Med Sci*. 2010;2(5):241-245. doi:10.4297/najms.2010.2241

31. Duraiyan J, Govindarajan R, Kaliyappan K, Palanisamy M. Applications of immunohistochemistry. *J Pharm Bioallied Sci*. 2012;4(Suppl 2):S307-S309. doi:10.4103/0975-7406.100281

32. Dako_Handbook.pdf. Accessed May 13, 2020. http://www.ihcworld.com/_books/Dako_Handbook.pdf

33. Technical Aspects of Immunohistochemistry - J. A. Ramos-Vara, 2005. Accessed May 13, 2020. https://journals.sagepub.com/doi/full/10.1354/vp.42-4-405

34. Immunofluorescence - an overview | ScienceDirect Topics. Accessed May 13, 2020. https://www.sciencedirect.com/topics/immunology-and-microbiology/immunofluorescence

35. IHC Detection. Novus Biologicals. Accessed May 23, 2020. https://www.novusbio.com/ihc-detection

36. Isom MM. tau-protein. In: *Store norske leksikon*. ; 2019. Accessed June 3, 2020. http://snl.no/tau-protein

37. Chromogenic immunodetection in IHC | Abcam. Accessed May 23, 2020. https://www.abcam.com/kits/chromogenic-detection-in-ihc

38. Chromogenic Detection for Western Blot, IHC, and ELISA. Accessed May 13, 2020. https://www.jacksonimmuno.com/secondary-antibody-resource/immuno-techniques/chromogenic-detection-for-western-blot-ihc-and-elisa/

39. Wojtowicz JM, Kee N. BrdU assay for neurogenesis in rodents. *Nat Protoc*. 2006;1(3):1399-1405. doi:10.1038/nprot.2006.224

40. Dzulkifli FA, Mashor MY, Jaafar H. An overview of recent counting methods for Ki67 IHC staining. *J Biomed Clin Sci JBCS*. 2019;3(2):10-17. Accessed June 1, 2020. http://apps.amdi.usm.my/journal/index.php/jbcs/article/view/180

41. Scholzen T, Gerdes J. The Ki-67 protein: from the known and the unknown. *J Cell Physiol*. 2000;182(3):311-322. doi:10.1002/(SICI)1097-4652(200003)182:3<311::AID-JCP1>3.0.CO;2-9

42. Zeng C, Pan F, Jones LA, et al. Evaluation of 5-ethynyl-2'-deoxyuridine staining as a sensitive and reliable method for studying cell proliferation in the adult nervous system. *Brain Res*. 2010;1319:21-32. doi:10.1016/j.brainres.2009.12.092

43. Cell proliferation: a complete guide | Abcam. Accessed June 1, 2020. https://www.abcam.com/primary-antibodies/the-cell-proliferation-guide

44. Massey AJ. Multiparametric Cell Cycle Analysis Using the Operetta High-Content Imager and Harmony Software with PhenoLOGIC. Cotterill S, ed. *PLOS ONE*. 2015;10(7):e0134306. doi:10.1371/journal.pone.0134306

45. BrdU Antibody (B35128). Accessed May 13, 2020. https://www.thermofisher.com/antibody/product/BrdU-Antibody-clone-MoBU-1-Monoclonal/B35128

46. BrdU control slides (IHC) (ab129956) | Abcam. Accessed May 13, 2020. https://www.abcam.com/brdu-control-slides-ihc-ab129956.html

47. Kee N, Sivalingam S, Boonstra R, Wojtowicz JM. The utility of Ki-67 and BrdU as proliferative markers of adult neurogenesis. *J Neurosci Methods*. 2002;115(1):97-105. doi:10.1016/S0165-0270(02)00007-9

48. Eminaga S, Teekakirikul P, Seidman CE, Seidman JG. Detection of markers of cell proliferation by immunofluorescent staining and microscopy imaging in paraffin-embedded tissue sections. *Curr Protoc Mol Biol*. 2016;115:14.25.1-14.25.14. doi:10.1002/cpmb.13

49. (6) (PDF) Pre- and Posttreatment With Edaravone Protects CA1 Hippocampus and Enhances Neurogenesis in the Subgranular Zone of Dentate Gyrus After Transient Global Cerebral Ischemia in Rats. ResearchGate. Accessed June 3, 2020. https://www.researchgate.net/publication/268227820_Pre-_and_Posttreatment_With_Edaravone_Protects_CA1_Hippocampus_and_Enhances_Neurogenesis_in_the_Subgranular_Zone_of_Dentate_Gyrus_After_Transient_Global_Cerebral_Ischemia_in_Rats

50. Merckmillipore. EdU Cell Proliferation Assay EdU-488 Catalog No. 17-10525 EdU-555 Catalog No. 17-10526 EdU-594 Catalog No. 17-10527 EdU-647 Catalog No. 17-10528. https://www.merckmillipore.com/SE/en/search/edu?search=&TrackingSearchType=SB+-+homepage-search-box+-+OLD&SearchContextPageletUUID=&SearchTerm=edu. Accessed June 2, 2020. https://www.merckmillipore.com/SE/en/product/EdU-Cell-Proliferation-Assay-EdU-488,MM_NF-17-10525

51. EdU Cell Proliferation Assay (EdU-488) | 17-10525. Accessed June 3, 2020. https://www.merckmillipore.com/SE/en/product/EdU-Cell-Proliferation-Assay-EdU-488,MM_NF-17-10525

6.0 Appendixes

Appendix I: Preparation and Solutions for BrdU-staining

Preparations, part I (max 5 days prior to staining)

Phosphate buffer (PB) 0,4M – pH 7,4

A (acid): 13,8 g sodium dihydrogen phosphate ($NaH_2PO_4*H_2O$) / 250ml H_20

B (base): 17,8 g Sodium hydrogen phosphate dihydrate ($Na_2H_2PO_4*2H_2O$) / 250ml H_2O

Make solutions A and B. Add solution A to solution B until Sol.B is at pH 7,4.

Store in a dark place at room temperature for up to one month.

Phosphate buffer 0,125M – pH 7,4

Dilute 0,4M Phosphate buffer until you have 250ml 0,125M. To achieve 250ml of 0,125M buffer solution add 73ml 0,4M PB to 172ml of H_2O.

Store at 4°C for up to one week.

Purpose of phosphate buffer

The purpose of the phosphate buffer is to maintain the osmolarity of the cells after fixation.

Tris-buffered saline (TBS) - pH 8,0

1500ml: 9,09g Tris + 13,44g NaCl / 1500ml H_2O

Adjust pH to 8,0 with HCl (2,0M).

Store in refrigerator for up to one week.

Purpose of Tris-buffered saline

TBS is an antigen retrieval solution used with paraffin embedded / formalin or paraformaldehyde-fixed tissues. When tissues fixed in cross-linking agents such as paraformaldehyde, these agents will covalently cross-link proteins, resulting in a reduction in the available epitopes for antibody binding. Use of the basic antigen retrieval solution effectively unmasks some proteins epitopes. *(https://www.protocolsonline.com/histology/immunohistochemistry-histology/tris-buffered-saline-tbs-antigen-retrieval-protocol/) lest: 24.03.20*

TBS-Tx (0,5%) - pH 8,0

500 ml: Under a ventilated hood, add 2,5ml Triton X-100 and mix well.

Store in a refrigerator for up to one week.

Purpose of addition of Triton X-100

Triton X-100 is a non-ionic detergent that disrupts lipid interactions in the cell- and nucleus membrane while leaving protein interactions intact. *(https://www.sciencedirect.com/topics/agricultural-and-biological-sciences/triton-x-100, read: 23.03.20)*

Tris-HCl – pH 7,6

250ml: 1,52g Tris / 250ml H_2O.
Adjust pH to 7,6 with HCl (2,0M).
Store in refrigerator for up to one week.

HCl 1M

50 ml: 4,2ml 37% HCl added to 45,9ml of H2O.
Do not make the mistake of adding water to concentrated HCl.
Store at room temperature in a well-ventilated place. Can be stored for an extended period of time.

HCl 2M

50 ml: 8,35 ml 37% HCl added to 41,7 ml of H2O.
Do not make the mistake of adding water to concentrated HCl.

Appendix II: BrdU protocol for neurogenesis in rodents

Day 1

(5 hours)

Note: Carry out procedure with sections in a 6-well plate with strainers. All rinsing and incubations should be performed on a plate shaker in room temperature, unless otherwise stated. Step 11 and beyond should be performed in the dark.

	Steps	Function
1	Carefully transfer brain sections to strainers using a soft paint brush.	
2	Rinse sections 3 x 5 min in TBS while still in the strainer.	Function of TBS is described under preparations.
3	Preheat 1M HCl to 45°C in a heating cabinet. Make sure there is between 500-1000ul in each Eppendorf tube.	
4	Denature DNA by incubating in the preheated 1M HCl-solution for 30 minutes at 45°C on a plate shaker in a heating cabinet.	DNA needs to be denatured to allow binding of antibodies in the tissue. This is achieved by exposing the tissue to both acid and heat in this protocol. This is a critical step as incomplete denaturation of DNA can lead to false negative results.
5	Rinse sections 3 x 5 min in TBS in a strainer.	
6	In a large petri dish, add the sections to PB 0,125M for 2 hours at 60°C	
7	Rinse sections 2 x 10 min in PS (in a strainer)	Neutralizes the use of PB

8	Rinse sections 1 x 10 min in TBS-Tx (in a strainer)	
9	Incubate sections with 10% goat serum in TBS-Tx for 60 minutes	Blocking solution. To prevent nonspecific binding.
10	Free-floating sections on plate shaker	
11	Add sections to glass slides (80 ul) Draw a circle around the section with a Dako pen	
12	Draw off excess solution (do not wash, just remove excess)	
13	On a plate shaker in tubes, incubate sections with primary antibody, Anti-BrdU antibody, 1:10 in TBS-Tx overnight at 4°C	Anti-BrdU binds to the BrdU in the tissue sections.
14	For control sections on glass slides: Incubate in a humidity chamber on top of two plastic pipettes, with wet paper towels laid down at the bottom. For free-floating sections, use an Eppendorf tube.	

Day 2

(2,5 hours)

15	Rinse sections 3 x 10 minutes in TBS-Tx Carefully extract the sections from the strainer using a paint brush	

16	Incubate sections in a secondary antibody, Goat anti-mouse IgG H&L Alexa Flour 488, 1:100 in TBS for 2 hours	
17	Cover tubes with aluminium foil	Should not be exposed to light.
18	Rinse sections 2 x 5 minutes in TBS	
19	Cover plate shaker with aluminium foil	
	Mount and coverslip on a glass slide	
20	When ready to mount sections, float in a petri dish with TBS and arrange orientation of sections	
21	Carefully transfer sections to slides using a paint brush and leave the glass slide at an angle to dry for at least 30 minutes	
22	Add 1 large drop of Vectashield and place coverslips over it, without pressing down	Liquid “fixation” medium.
23	Seal the coverslip with nail polish(?) and let it dry in the dark	Nail polish seals the edges and makes sure the Vectashield-solution does not dry out.
24	Mounted tissues can be stored for up to 6 months in a refrigerator at 4°C	

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