Regulating synaptic protein expression by *in vivo* antibody treatment to reduce epileptogenesis phase in rats

LUND INSTITUTE OF TECHNOLOGY
Lund University

Student: Vedran Matinjanin, fys09vma@student.lu.se, +46 707230493
Supervisor: Doc. Christine Ekdahl Clementson, christine.ekdahl_clementson@med.lu.se,
Examiner: Prof. Leif Bülow, leif.bulow@tbiokem.lth.se, +46 46 2229594
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Abstract

Epilepsy is a neurological disorder characterized by its debilitating epileptic seizures. There are generalized seizures, which affect the whole brain at once, and focal seizures, which start from one part of the brain. The most common focal epilepsy, is temporal lobe epilepsy representing almost 70% of all focal epilepsies. Around 30% of all patients are therapy refractory, therefore there is a huge demand to find new drugs and ways to help all patients with epilepsy. It has been previously found that synaptic proteins play a crucial role in epilepsy, and in previous studies the protein N-cadherin has been found to be decreased 4 weeks after induced epilepsy in rats that did develop spontaneous seizures. In this study we set out to modulate n-cadherin protein expression with antibodies in order to evaluate if this could potentially be a novel method to reduce seizure frequency and seizure-induced cognitive deficits. For a more clinically realistic scenario some animals were given a combinatorial treatment of the antibody and an anti-epileptic drug named Keppra to see if any synergistic effect can be achieved. In the first pilot study we infused N-cadherin antibody for 1 and 2 weeks to study the antibody distribution and suitable antibody concentration that would not induce overt morphological changes. In addition, we evaluated the behaviour of the rats. The preliminary results show that rats induced with 1000µg/ml antibody over the course of two weeks show no signs of bad health, although they had a tendency to be more anxious than the untreated rats. Future studies will reveal if N-cadherin antibody treatment has any effects on seizure frequency.

Preface and Acknowledgements

This project has been challenging and enlightening at the same time. I have learned a lot through mistakes and hard work, and I would like to extend a special thank you to professor Bülow, who has guided me and lit the spark in me with his inspiring and stimulating lectures. Furthermore, I would like to thank docent Christine Ekdahl Clementson for taking me in and giving me a challenging project, and a big thanks to her PhD students Una Avdic and Matilda Ahl for helping me through my clueless times and teaching me everything I had to know to finish at the end of the line. Finally I would like to thank my family, who have always supported me no matter what decision I have made and always made me feel at home in their hearts.

Introduction

Epilepsy

Epilepsy is a chronic disorder of the brain that is characterized by recurrent epileptic seizures and affects people all around the world. In the United States alone, it is estimated that 1 in 26 people will develop epilepsy during their lifetime (Epilepsy, 2014) which makes it the fourth most common neurological problem with migraine, stroke, and Alzheimer's disease occurring more frequently. As of 2015 there were about 39 million people with reported epilepsy (GBD 2015 Disease and Injury Incidence and Prevalence Collaborators, 2016). The seizure severity can vary from small lapses of attention to severe and prolonged convulsions and muscle jerks and the frequency may vary as much as having several seizures per day to less than one seizure per year (WHO, 2016). The seizures can be categorized into two different groups, partial and generalized seizures. The partial seizures start in a specific part of the brain, often in the temporal lobe or specifically in the hippocampus (HPC). The symptoms of TLE includes confusion, altered mental status, chewing, drooling or automatism. Generalized seizures on the other hand start in both hemispheres at the same time and can be observed as loss of consciousness and often manifests as convulsive, tonic-clonic events. The generalized seizures require high metabolism and if the seizures are not stopped, life threatening symptoms such as cardiac or renal failure or electrolyte imbalance may be triggered (Tatum,2001). Epilepsy is diagnosed by electroencephalogram (EEG) which detects electrical activity on top of the brain scalp and magnetic resonance imaging (MRI), which uses strong magnetic fields, radio waves, and field gradients to form an image, to look at the structure of the brain. A person is diagnosed with epilepsy when they have had two unprovoked seizures, which are seizures that are not caused by a known and reversible medical condition such as alcohol withdrawal, low blood sugar, or sleep deprivation. Furthermore, patients who have remained seizure-free for the last 10 years with no medication for the past five years are considered to be resolved of their epilepsy (Fisher, 2014).

The most common form of partial epilepsy is temporal lobe epilepsy (TLE) and it is commonly drug resistant. The affected area of the brain is often the hippocampus, which is an important component of the brain in humans and other vertebrates, playing a crucial role in forming long-term memories from short-term memories and in spatial memory, which enables navigation. There are two hippocampi, one in each brain hemisphere, in humans and other mammals and belong to the limbic system which support a variety of functions including emotion, behavior, motivation and long term memory (Martin, 2003). The hippocampus is located under the cerebral cortex and contain two main parts, the hippocampus proper and the dentate gyrus. The hippocampus has the shape of a curved tube, resembling a seahorse or a ram's horn (Cornu Ammonis) in which the abbreviation CA comes from when naming the hippocampal subfields; CA1, CA2, CA3, and CA4. The dentate gyrus consists of different cell types which are both excitatory and inhibitory, the primary excitatory cells receive information from outside of the hippocampus, including entorhinal cortex. The dendrites receive the information and transport it to the rest of the hippocampus via the axons of the granual cells.

Therapy

There is available medication to manage the seizures and the drugs are most often anticonvulsant (NICE 2012) also called anti-epileptic drugs (AED), although the medication is only effective on 70% of all patients (Eadie, 2012). However, the efficacy of medication on patients with partial or focal epilepsy is only around 50% (Bergey, 2013). Most treatments involve modulating the activity of γ-aminobutyric acid (GABA) receptors or reducing the glutamatergic transmission, both of which are used inhibitory and excitatory synapses respectively. For those patients that are resistant to medication there is a last resort surgery which is the resection of the part of the brain which the seizures originate from (Duncan 2007). There are around 26 AEDs used in the treatment of seizures with different drugs treating different types of seizures (Epilepsy Society, 2014). For example, the drug Levetiracetam, which goes under the generic name of Keppra, is used in this study. The exact mechanism of levetiracetam is unknown, however, the drug binds to a synaptic vesicle glycoprotein *SV2A* (Lynch 2004) and inhibits presynaptic calcium channels (Vogl, 2012). Sometimes one drug is not enough to stop the seizures therefore combination of drugs is often utilized.

Epileptogenesis

The exact mechanism of epilepsy is unknown, however, excitatory neurons are believed to become more active and hyperexcitable (Goldberg, 2013). Epilepsy can be categorized into two groups, genetic/idiopathic and acquired. In idiopathic epilepsy the cause is not known but is usually associated with a genetic component and may include mutations of genes responsible for maintaining the excitatory/inhibitory (E/I) balance in the brain, eg ion channels as well as environmental factors can play a role. Acquired epilepsy on the other hand includes a precipitating injury or brain insult such as traumatic brain injury, including a brain infection, tumour, ischemic stroke or cortical dysplasia. These brain insults trigger a series of events that reorganize the brain network and make it more prone to developing spontaneous recurrent seizures. This process is termed epileptogenesis and can last from months to years. More specifically, aberrant molecular and cellular plasticity, neurogenesis, inflammation, neurodegeneration, neurochemical changes occur, which in combination or alone cause the inhibitory and excitatory circuits to reorganize which in turn lowers the seizure threshold (Pitkänen, 2002).

One of the mechanisms thought to be important in epileptogenesis is the E/I balance in the brain. Synaptic proteins and scaffolding proteins that sit on the pre- and postsynaptic sites, presynaptic sites are the ones that send the message and postsynaptic sites are the ones that receive the message, on neurons are thought to play an important role in maintaining this balance (Chugh, 2015). Previous studies have indicated altered expression in synaptic proteins in both patients and animal models of epilepsy. It has been recently found that synaptic proteins are altered in the brain at different time points following status epilepticus (SE). Interestingly, at 4weeks following SE, the excitatory adhesion molecule N-cadherin was decreased in animals that had developed epilepsy, whereas no change in expression was detected in animals that did not develop epilepsy following the insult (Avdic, 2017, *manuscript*). N-cadherin is found in neurons at both pre- and postsynaptic terminals. Each N-cadherin has a small part in the cytoplasm, that is anchored to the membrane and the rest of it is extracellular, which is the part that is used to adhere to other cadherins. The intracellular domain is connected to the

cytoskeleton by binding to beta-catenin molecules. N-cadherin is important due to its mediation of neuronal and growth cone migration when N-cadherin/beta-catenine links to actin in the early stages of development. In the later stages of synaptic development N-cadherin becomes more concentrated at excitatory synapses and it has been shown that a loss of N-cadherin at excitatory synapses has decreased the severity of kainic acid induced hippocampal seizures (Niikitczuk, 2014).

Models of acquired epilepsy

Electrical models
Kindling is an animal model used to mimic the symptoms of TLE and is widely used. The model applies repeated short electrical stimuli to hippocampus or amygdala over a long time period which results in a progressive increase in electrographic and behavioural seizure activity (Goddard, 1967). This model shares similarities with the human condition in terms of semiology and pharmacology used to help with the seizures (Loscher, 2002).

In addition to kindling, a more acute model is used where electrical stimulation in hippocampus or amygdala develop SE that becomes selfsustained, no stimulation is needed for seizures to occur. Animals induced with SE get spontaneous recurring seizures and neuropathological changes including hippocampal sclerosis, severe neuronal cell loss also found in many patients with TLE.

Chemical models
Chemical compounds such as pilocarpine, which is a cholinergic agonist, and kainic acid,a glutamate analog, can induce SE (Loscher, 2002) and is administered either systematically or locally in the brain. Most animals develop spontaneous recurring seizures after a latent period of weeks to months.

Traumatic brain injury models
Some models want to mimic the human epileptic condition developed after a traumatic brain injury. These models include fluid-percussion, controlled cortical impact or ballistic injury models. All models use mechanical damaged to the brain and these animal models often have a long duration to the development of spontaneous seizures and the seizure frequency is substantially lower (Pitkänen, 2009).

Aim

The change in N-cadherin expression that we see at 4weeks post SE may reflect a compensatory mechanism in order to restore the E/I balance. Therefore, the aim of this project was to target N-cadherin with an antibody and modulate its expression in the brain in order to alter the epileptogenic state and hopefully affect the seizure frequency.

Materials and Methods

Animals

Adult male Sprague-Dawley rats weighing between 200-250 g were procured from Charles River (Germany). The animals were housed in 12 hrs light/dark cycle with ad libitum food and water. All experimental procedures followed the guidelines set by the Malmö-Lund Ethical Committee in Sweden for the use and care of laboratory animals.

Group assignment

Animals were divided into five groups shown in table 1 below.

Table 1: Table showing planned animals group assignment followed by the number of animals

Group	Total number of animals
N-Cad Concentration Determination	7
Keppra	6
SE Control	6
Non-stimulated control (NSC)	9
N-Cad + Keppra	6
Total	34

All animals had electrode, cannula and pump implanted.

Surgeries, drug infusions and electrically-induced temporal status epilepticus

Animals were anaesthetized with 2% isofluorane and implanted with a bipolar insulated stainless steel electrode (Plastics One, Roanoke, VA) into the right ventral CA1/CA3 region of the hippocampus (coordinates: 4.8 mm posterior and 5.2 mm lateral from bregma; and 6.3 mm ventral from dura, tooth bar set at -3.0 mm) for stimulation and recording. A unipolar electrode was placed between the skull and adjacent muscle to serve as ground electrode. Additionally, a brain infusion cannula (Brain infusion kit 1, Alzet) was implanted in the lateral ventricle (coordinates: 1.0 mm posterior and 1.5 mm lateral to bregma; and 3.5 mm ventral to the flat skull position with bregma as reference) in the ipsilateral hemisphere. Following a week of recovery after surgery, rats were subjected to electrically-induced temporal SE. Afterdischarge threshold was assessed by delivering a 1 s train of constant current balanced, biphasic, square-wave pulses (1 ms duration, 50 Hz) at an initial current of 10 µA (base-to-peak), and increased by 10 µA increments at 1 min intervals until at least a 5s duration of afterdischarge was evoked. After 30 min, a 1 hour suprathreshold stimulation with 10 s trains of 1 ms, 50 Hz, biphasic, square-wave pulses was applied. Stimulation was interrupted every 9th min for 1 min of EEG recordings. Electrode-implanted non-stimulated rats served as controls (NSC). Only rats that displayed self-sustained EEG activity for 2 hours in the temporal lobe and mainly partial seizures e.g. orofacial twitches, nodding, drooling, and unilateral forelimb clonus, according to Racine´s scale, were included in this study (Racine 1972). Behavioural symptoms and EEG activity was completely interrupted after 2 hours of self-sustained SE by administration of pentobarbital (65 mg/kg, intraperitoneal injection).

Intracerebroventricular drug infusion

After being anesthetized following SE, the osmotic pump was connected to the brain infusion cannula. Non-stimulated control rats were also connected with the pumps, only carrying vehicle. The osmotic pumps were placed in the subcutaneous pocket in the dorsal region of the neck. For the concentration determining part 1 week pumps (2001 1.0µl/hr, Alzet) were used and for the treatment 2 week pumps (2002 0.5µl/hr, Alzet). Table 2 shows the pumps and the concentrations used in the pilot study.

Table 2: Table showing concentrations of treatment and following pump specs used for each treatment.

Concentration [µg/ml]	Model	Reservoir Volume [µl]	Pump Flow [µl/hr]
10	2001	200	1.0
80	2001	200	1.0
200	2001	200	1.0
300	2001	200	1.0
500	2001	200	1.0
1000	2002	200	0.5

Western Blot

In order to see if any N-cadhein was cleaved during the treatment and was present in the blood we performed western blot analysis on serum from animals treated with N-cadhein antibody. Cardiac blood was collected from animals before perfusion and incubated in room temperature for 1h. The saples were then centrifuged at 2000rpm for 30min, 4°C. The supernatant was collected, aliquoted and stored in -80°C until analysis.

Protein samples were denatured at 99°C for 5 min in 2x Laemmli sample buffer (Biorad, Germany). Total protein of 10–20 µg (unless otherwise mentioned) was resolved on precast 4–15% mini-PROTEAN TGX (Biorad) sodium dodecyl sulphate polyacrylamide gels and transferred using Trans-Blot Turbo mini nitrocellulose transfer packs (Biorad). Following this, the membranes were blocked for 2 h at room temperature in tris-buffered saline (pH 7.4) with 0.2% (w/v) Tween 20 (TBS-T) containing 5% nonfat dried milk. Membranes were then incubated overnight at 4°C with primary antibodies diluted in TBS-T containing 0.5% bovine serum albumin (BSA) (Sigma). The only primary antibody used was Monoclonal Anti-N-Cadherin antibody produced in mouse purified from hybridoma cell culture (1:200 Sigma Aldrich, Germany). After washing, membranes were incubated with secondary antibody diluted in TBS-T containing 0.5% BSA for 2 h at room temperature. Secondary antibody used was horseradish peroxidase-conjugated anti-mouse (1:1000 Sigma Aldrich). The membranes were then washed three times in TBS-T. Immunoreactive bands were subsequently visualized by enhanced chemiluminescence (Biorad), and images were acquired using Chemidoc XRS+ system (Biorad).

Immunohistochemistry

For analysis of adhesion molecules and inflammatory markers, rats were given an overdose of pentobarbital (250 mg/kg i.p.) and transcardially perfused with ice-cold 0,9% saline and ice-cold PFA (4% in 0.1 M PBS, pH=7.1-7.4). Brains were removed, post-fixed in 4% PFA overnight, and then dehydrated in 20% sucrose in 0.1 M PBS overnight, and then cut into 30 µm-thick coronal sections using a microtome. The brains were marked in the right hemisphere

by a cut in cortex to determine which is left and right when examining. The brain was glued to a fixed holder using a cryomold (*Tissue-Tek O.C.T Compound and Cryomolds*) which hardens at -10°C, using dry ice, and fixates the brain for better sectioning. Free-floating sections were incubated with the appropriate primary antibody overnight at +4°C and secondary antibody for 2 h at room temperature. Following primary antibodies were used: mouse anti-N-cadherin (1:500 Sigma Aldrich, Germany), rabbit anti-IBA1 (1:500 Wako, Germany), and mouse anti-ED1 (1:200 Abd Serotech). Secondary antibodies used were: Alexa -488 donkey anti-mouse (1:200 Invitrogen, Sweden), biotynilated goat anti-rabbit (1:200 Vector Laboratories, UK), Alexa-488 conjugated streptavidin (1:200, Jackson ImmunoResearch, UK), and Cy3-conjugated donkey anti-mouse (1:200 Jackson Immuno Research). Free-floating sections from perfused animals were mounted on gelatin-coated microscope slides and coverslipped with DABCO (Sigma) and Hoescht (Sigma) 1:1000.

Statistical analysis
Statistical analyses were performed with unpaired Student's *t*-test when comparing two groups, using GraphPad Prism software and paired Student's *t*-test when comparing within a group.

Racine stages
In order to quantify epileptic seizures, Ronald J. Racine suggested means to describe seizure intensities. As the intensity of the seizures increase, so does severity. All stages are a result of the action potentials causing the muscles to contract and relax, resulting in an involuntary and observable action (Racine, 1972).

Stage 1, mouth and facial movements twitching
Stage 2, head nodding
Stage 3, forelimb clonus
Stage 4, rearing with forelimb clonus
Stage 5, rearing and falling with forelimb

Behavioural tests
In order to assess the cognitive function of memory, anxiety, and motor functions behaviour tests were conducted.

Open Field
Open field is used to measure locomotor activity and anxiety in individual rats. The field is quadratically formed with the dimensions 90cmx90cm divided into 16 squares 15cmx15cm and walls with the height of 40cm. The following parameters were measured each minute, time spent moving/not moving, grooming (yes or no), rearing (yes or no), time spent in the middle quadrant, and number of boxes crossed.

Social Interaction
Social interaction is used to measure the wellbeing and anxiety of rats. A rat with no surgical history was introduced to the rat being tested and the parameters measured each minute where, time spent interacting, total numbers of interactions, and if the testing rat is dominant or submissive.

Sugar Preference test

Sugar preference test is used to measure anhedonia and depression. After a period of 6 hours without water two bottles were introduced to the rats, in which one has 2% glucose. The parameter looked at were the intake of the sugar water compared to total liquid intake.

Y-maze

Y-maze is used to measure the willingness of rodents to explore new environments and to assess memory. The maze is Y-shaped with three arms at 120° angle from each other. The rat was introduced into the middle of the maze and exploration of each arm is recorded. The definition of an entry into one arm was defined when all four limbs were within the arm. When the rats explored all three arms consecutively it was considered a spontaneous alternation performance (SAP) and when the rat revisited the previously visited arm it was considered an alternating arm return (AAR).

Cylinder Test

Cylinder test is used to measure motor and muscle functions and to evaluate a rats' spontaneous forelimb use. The rat was put inside a glass cylinder for exploration. The parameters measured were the total usage of left and right forelimb each minute.

Results

Concentration determination

In order to determine a suitable antibody concentration for intracerebral treatment, a low and high dose were decided. The first concentrations tested were 10 and 80 µg/ml. After one week of antibody treatment immunohistochemistry on coronal slices using only secondary antibody, revealed no n-cad specific fluorescence binding to treatment antibody was found. As negative control, animals which also had pumps, although only containing KPBS, were stained with only secondary antibody. Figure 1 shows the lack of specific fluorescence staining around hippocampus and dentate gyrus with 80µg/ml treatment. All pumps were empty upon retrieval.
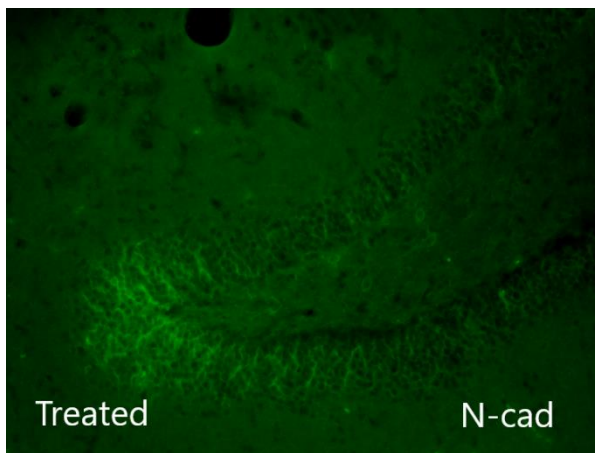

Figure 1: Hippocampus stain with treatment of 80µg/ml, lacking in staining showing mostly background.

Next, three different higher concentrations were tried, 200µg/ml, 300µg/ml, and 500µg/ml. Primary protein binding from the infused antibody were found after 300µg/ml and in 500µg/ml administered near the injection site. Figure 2 shows fluorescence staining at hippocampus with 300µg/ml and near the ventricle with 500µg/ml treatment.

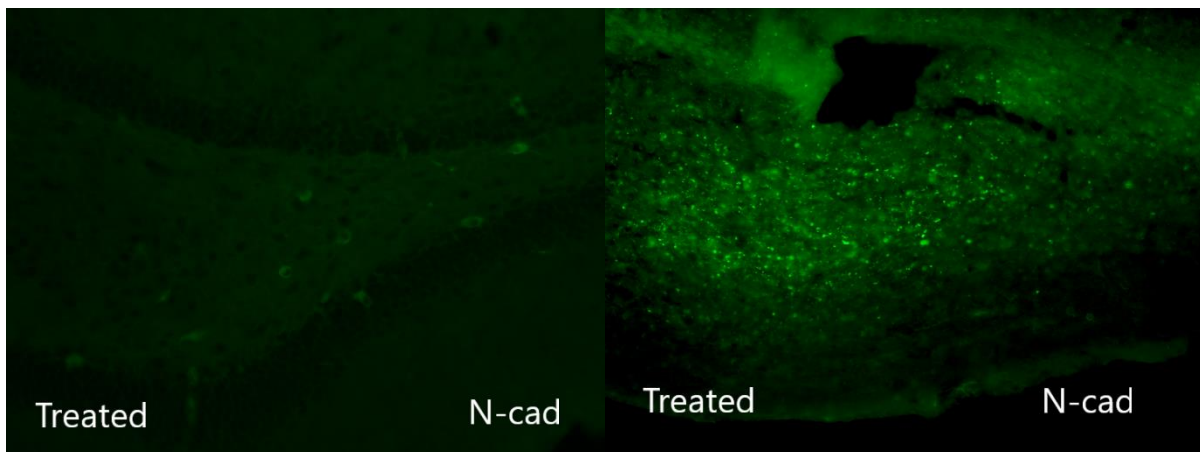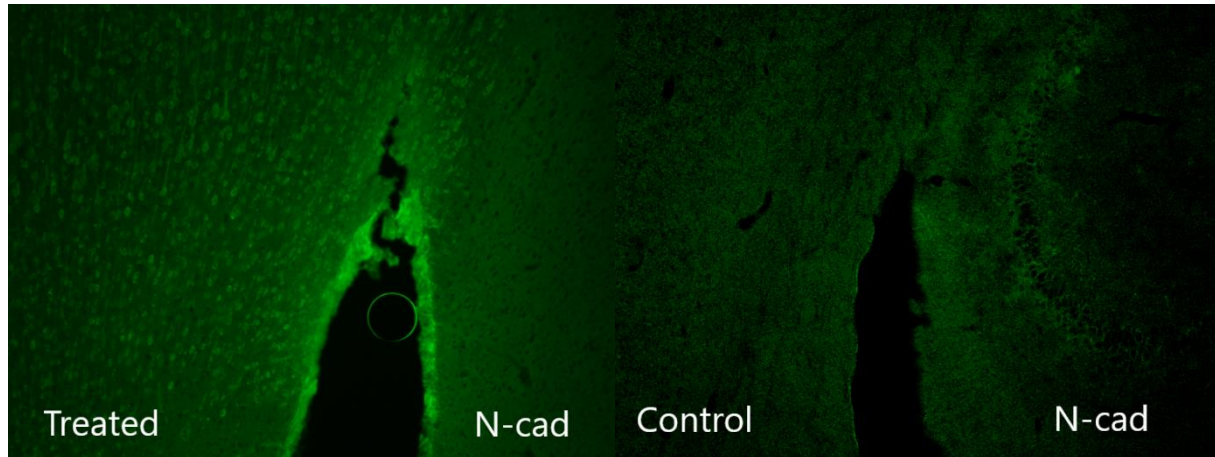*Figure 2: Left picture shows staining of the dentate gyrus with 300µg/ml treatment and right picture shows staining around the cannula site with 500µg/ml treatment.*

The concentration of 500µg/ml was deemed to be best suited for the treatment, due to its clearer staining. For the actual treatment, 2 week pumps are to be used instead of 1 week pumps. The infusion rate of the 2-week pump is half of the 1 week. In order to get the same amount of antibody in 2 the 2 weeks treatment, the concentration was doubled. The working concentration for the 2 weeks treatment was therefore determined at 1000µg/ml. Figure 3 shows the fluorescence staining near the ventricle of 1000µg/ml on treated and control rat.

Figure 3: Left picture shows staining around the ventricle with treatment of 1000µg/ml and right picture shows staining around the ventricle with no treatment.

Western Blot

Results from western blot showed no free n-cadherin in the blood.

Determination of toxicity of working concentration

To infuse 1000µg/ml antibody in the ventricle could cause damage and possibly lead to cognitive or behavioural deficiencies. To determine any alterations, several behaviour tests were conducted. The rats looked healthy, grooming them self and showing of shiny fur, and weighted within normal ranges, 300-350g.

Open Field

The open field test showed a significant decrease in N-cadherin treated rats with less time moving in the middle of the box compared to controls ($p < 0.05$ for 10 minutes, $p = 0.097$ for 3 minutes), which could indicate a higher level of anxiety. Although, the amount of time spent moving ($p = 0.99$ for 10 and 3 minutes) and the distance covered ($p = 0.61$ for 10 minutes, $p = 0.85$ for 3 minutes) detected no significant difference, indicating intact locomotive functions. Figure 4 displays the average time, and number of boxes crossed in treated and control rats.
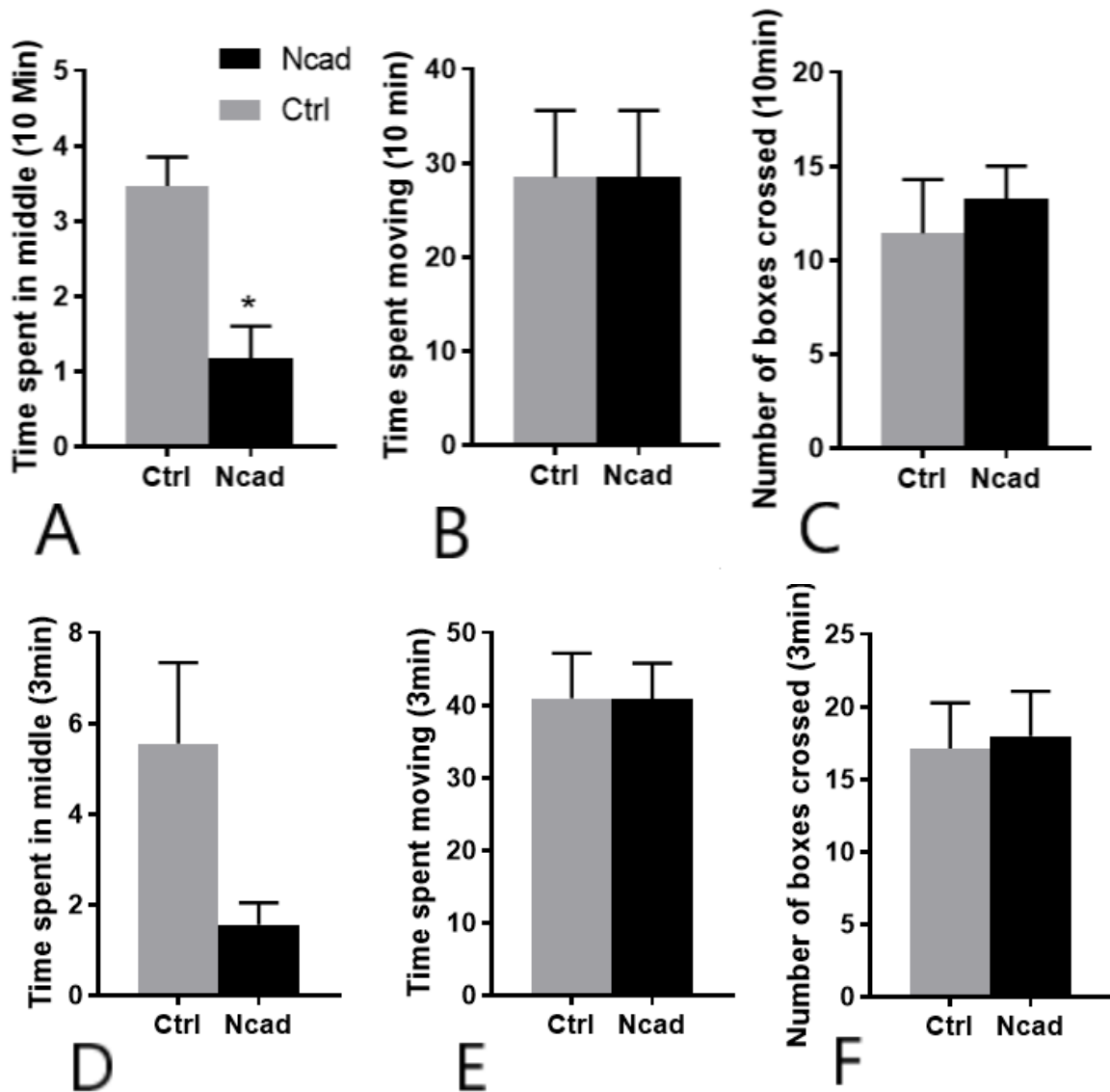

Figure 4: A: Comparison graph of average time spent in the middle of the field with a significant difference for a total of 10 minutes. B: Comparison graph of average time spent moving for a total of 10 minutes. C: Comparison graph of average number of boxes crossed for a total of 10 minutes. D: Comparison graph of average time spent in the middle of the field for a total of the first 3 minutes. E: Comparison graph of average time spent moving for a total of the first 3 minutes. F: Comparison graph of average number of boxes crossed for a total of the first 3 minutes.

Cylinder Test

The cylinder test shows no significant difference in activity (p = 0.48 for 10 minutes, p = 0.39 for 3 minutes) or predominant use of right or left paw (p = 0.32 for right paw comparison 10 minutes, p = 0.68 for left paw comparison 10 minutes, p = 0.34 for right paw comparison 3 minutes, p = 0.47 for left paw comparison 3 minutes between groups, p = 0.58 for left versus right 10 minutes within control group, p = 0.09 for left versus right 10 minutes within N-cadherin treated group, p = 0.51 for left versus right 3 minutes within control group, p = 0.18 for left versus right 3 minutes within N-cadherin treated group). Figure 5 shows graphs of left and right predominance between and within the groups.
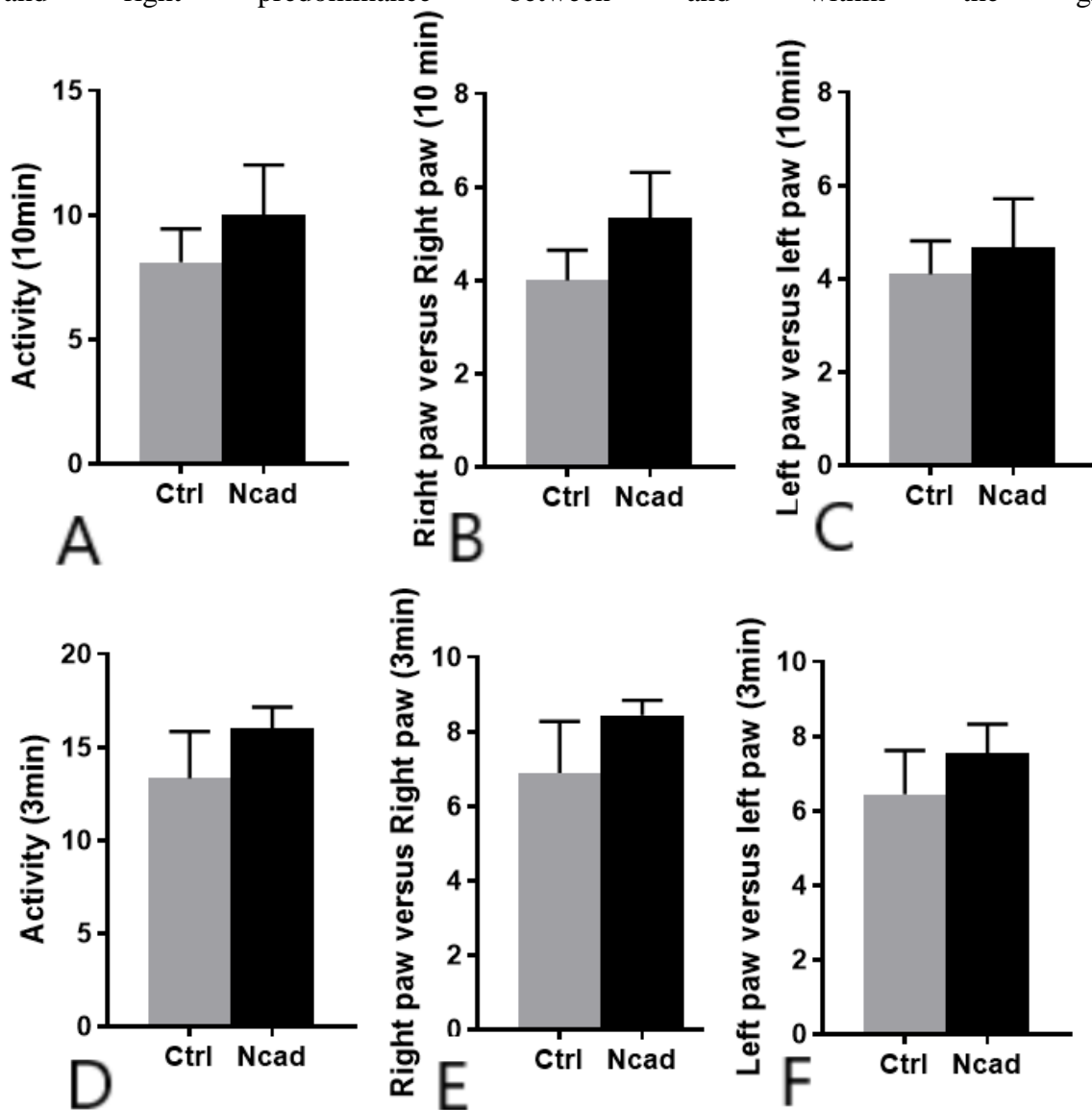

Figure 5: A: Comparison graph of average number of usage of paws for a total of 10 minutes. B: Comparison graph of average number of usage of the right paw for a total of 10 minutes. C: Comparison graph of average number of usage of the left paw for a total of 10 minutes. D: Comparison graph of average number of usage of paws for a total of 3 minutes
E: Comparison graph of average number of usage of the right paw for a total of 3 minutes. F: Comparison graph of average number of usage of the left paw for a total of 3 minutes

Social Interaction Test

The social interaction showed no significant difference in time spent interacting (p = 0.20 for 10 minutes, p = 0.13 for 3 minutes) nor in the amount of interactions (p= 0.43 for 10 minutes, p = 0.75 for 3 minutes) between control and N-cadherin treated rats. Although a trend towards decreased time spent interacting could be observed in treated rats, however since the groups included few annimals (n=3/group) further investigation to determine this is needed. Figure 6 compares time spent interacting and number of interactions between control and treated animals.
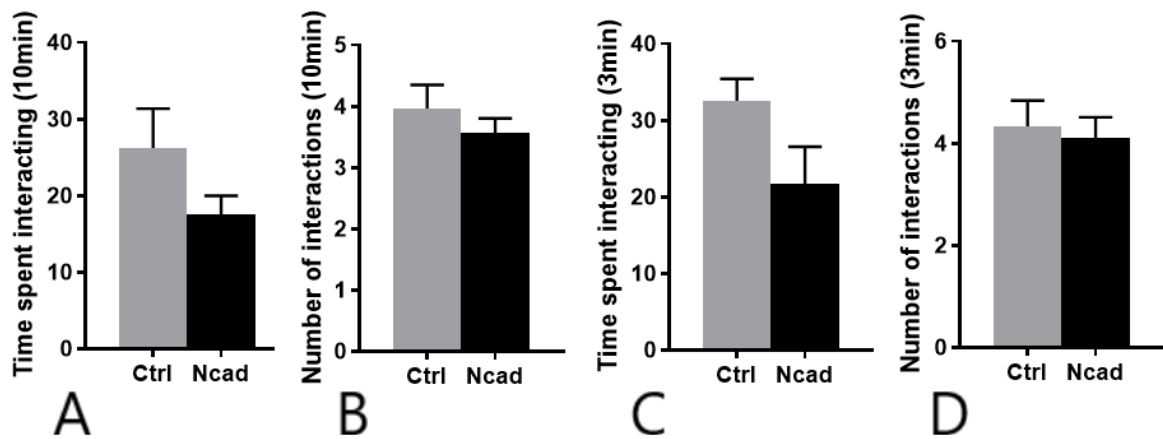

Figure 6: A: Comparison graph of average time spent interacting for a total of 10 minutes. B: Comparison graph of average number of interactions for a total of 10 minutes. C: Comparison graph of average time spent interacting for a total of the first 3 minutes. D: Comparison graph of average number of interactions for a total of the first 3 minutes

Y-maze

Y-maze showed no significant difference in spontaneous alternation performance (p = 0.53) and alternate arm return (p = 0.33). Figure 7 shows comparison graphs between groups.

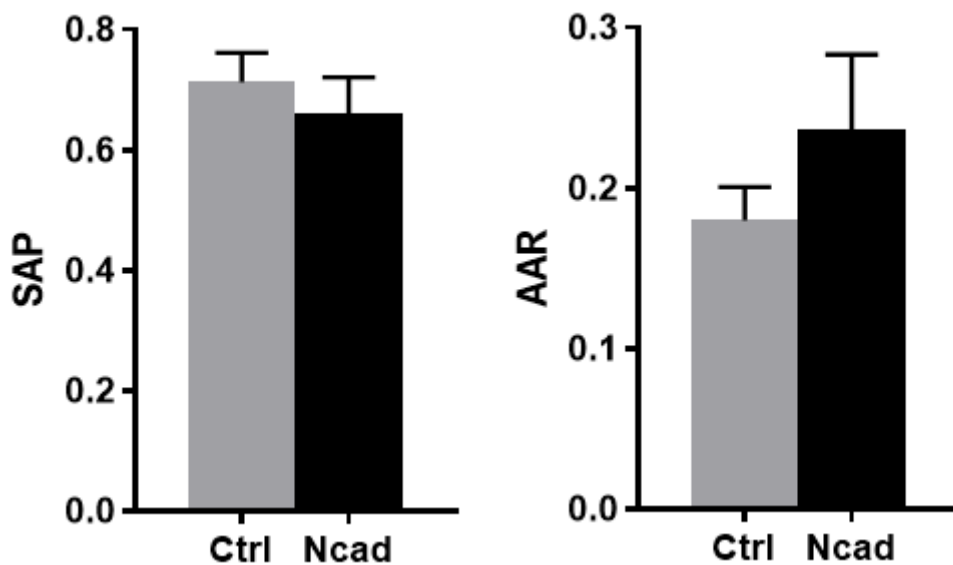*Figure 7: Left pictures shows comparison graph of spontaneous alternation performance and right picture shows comparison graph of alternate arm returns.*

Sugar Preference Test

The sugar preference test showed no significant difference in sugar preference (p = 0.57).
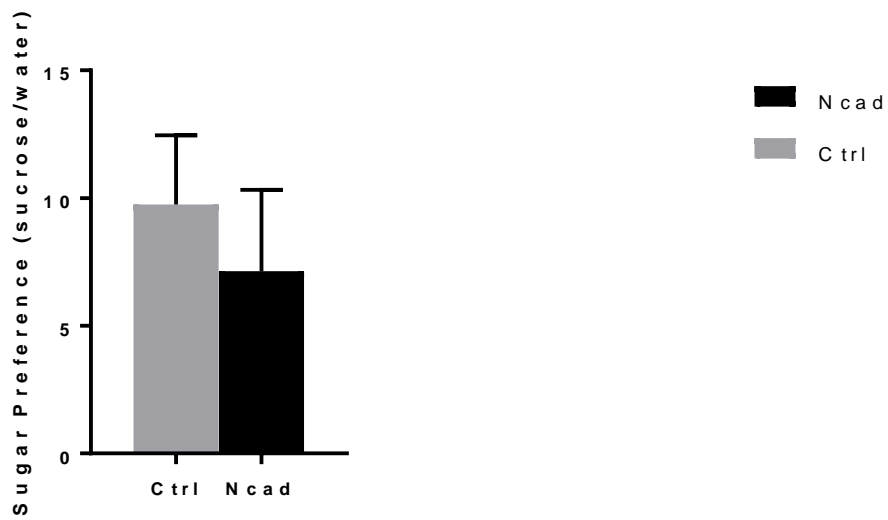Figure 8 shows comparison graphs between groups.

Figure 8: Comparison graph showing sugar preference in rats as a ratio sugar water : water.

Inflammatory markers

In addition to behaviour tests, immunofluorescence targeting one part glial population namely the microglia with IBA1 staining was done. Cells were counted to compare inflammation within hippocampus, from same side as the cannula was inserted (ipsilateral side). Cells were quantified in the granular cell layer (GCL), hilus, and in the molecular layer (ML) of the hippocampus. In Figure 9 and 10 the number of IBA1 positive cells in the hippocampus of control and treated rats are shown. No significant difference was found in the GCL (p = 0.14), hilus (p = 0.13), and in ML (p = 0.15).
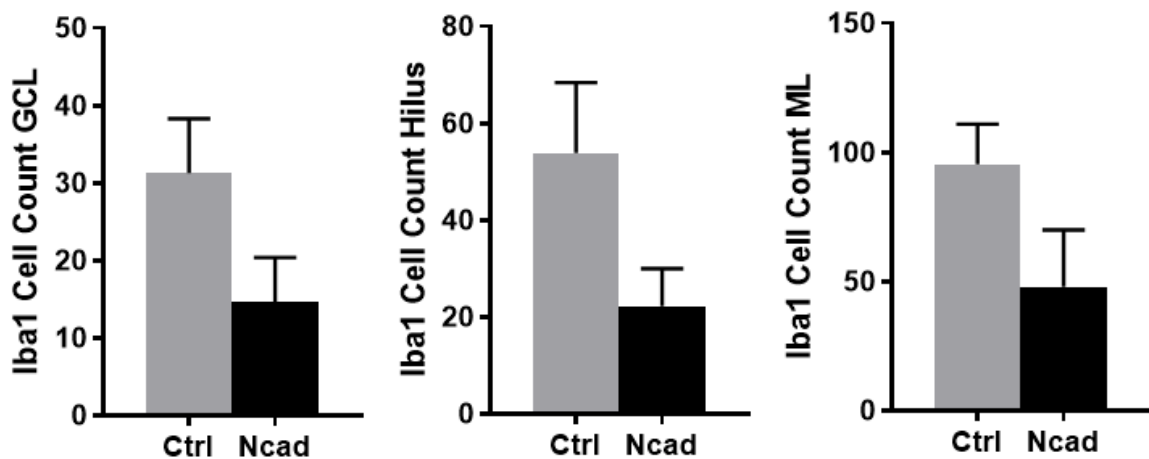

Figure 9: A: Cell count of Iba1 positive cells in the granular cell layer. B: Cell count of Iba1 positive cells in the hilus. C: Cell count of iba1 positive cells in the molecular layer.
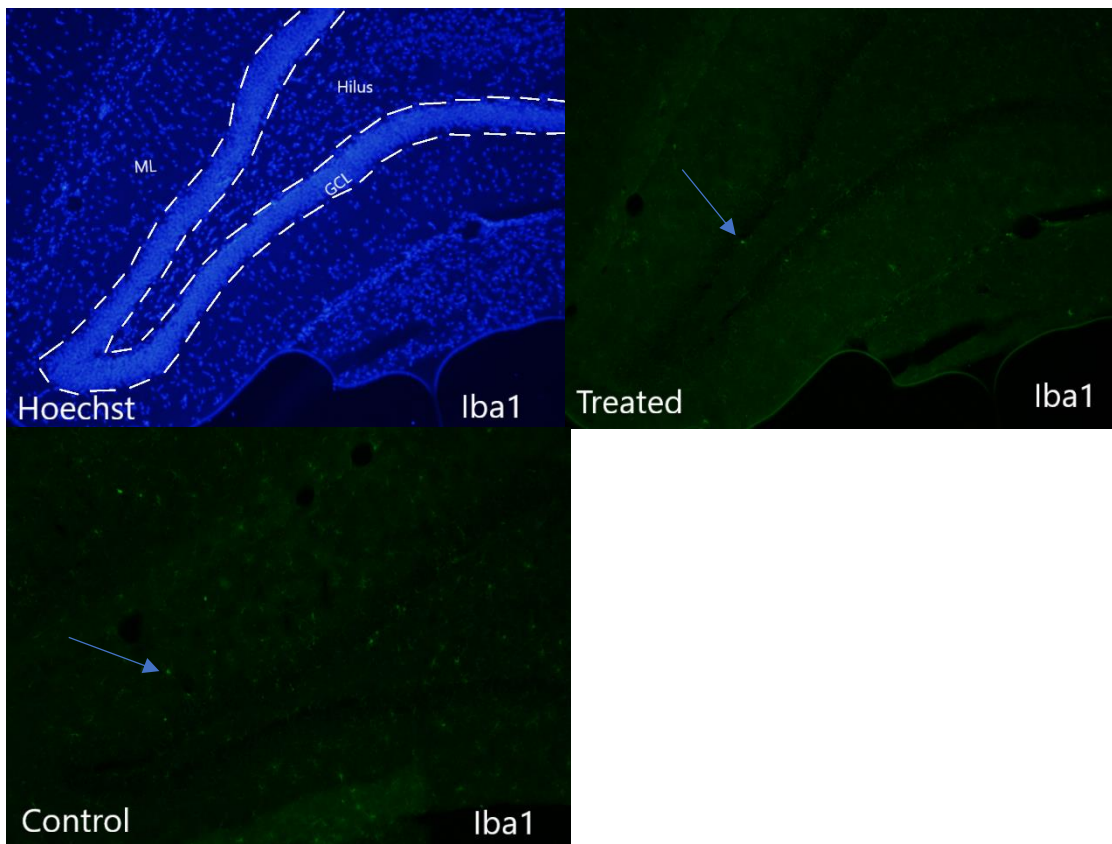

Figure 10: Iba1 fluorescence staining of the dentate gyrus of treated and control brain with arrows showcasing a typical cell counted.

Discussion

The pilot study resulted in a concentration that was detectable. However, it is not yet known if the same concentration has any therapeutic effects. The administration of 1000µg/ml concentration showed no health damaging effects, although behaviour tests indicated some anxiety.

Concentration Determination

The used antibody in this study is a Monoclonal Anti-N-Cadherin antibody produced in mouse purified from hybridoma cell culture. The antibody is a IgG1 antibody originating from mouse with specificity for N-cadherin in chicken, monkey, rabbit, human, mouse and rat. It is deemed suitable for applications like flow cytometry, electron microscopy, immunocytochemistry and western blot. Reason for using this specific antibody is due to it having an extracellular epitope and the buffer it is contained in did not contain any preservatives. However, we are using it in vivo which it has not been tested for and the animal being used is rat. Due to the antibody originating from mouse an adaptive immune response might be triggered rendering the antibody ineffective. The antibody should bind to the extra cellular domain and block adhesion, or even cause phagocytic cells to remove it, as well as bind to the N-cadherin in the brain. The amount needed to get a detectable amount is high, while the therapeutic effect concentration might be much lower. This is however something we cannot evaluate until it is administered in epileptic animals. Another issue with the staining could be that the secondary antibody used has trouble finding and binding to the primary antibody, or that the secondary antibody is old and the fluorophores are losing efficacy. This is combated by using fresh secondary antibody and the fact that N-cadherin is extracellular should be enough to make it detectable. These are some drawbacks that cannot be evaded due to the uncharted territory of this study, using antibody as a treatment for epilepsy.

Western Blot

The Western blot detected no bands for n-cadherin in the blood. This finding indicates that the cleaved n-cadherin is not transported in the blood or is at unmeasurable amounts which is hard to trace. It could also indicate that the antibody can't find its epitope due to the unfolding of the cleaved protein. No further evaluation of western blot was made due to this.

Determination of toxicity of working concentration

The behaviour tests conducted were all video recorded and analysed manually. To analyse videos by hand gives rise to a margin of error due to human factor, although most of the errors would be consistent throughout the tests. There is available technology which does the analyse which could reduce the error, however, this equipment is very expensive. Difference in day to day mood of the experimenter and sex could also play a role in the stress level of the rats although the effects could be negligible. Having multiple experimenters also gives rise to more deviations. Housing the rats alone in cages may affect the outcomes as rodents tend to become more depressed than if housed in a social environment, although all the rats in this study are caged alone which would affect all the rats in the same way.

Open Field

In the open field, rats were put in the middle of the box and recorded for 10 minutes. As time goes on and the rat familiarizes with the box, it stops to move around and starts feeling comfortable enough to start grooming. This gives a misrepresentation of the activity of the rat and is why all the measurements and comparisons are made in averages of 10 minutes and the

first   3   minutes   in   all   tests.
The treated rats in this study showed a significant difference ($p < 0.05$) in exploring the middle compared to control in the 10 minutes comparison, but showed only a trend ($p = 0.097$) in the first 3 minutes comparison. The activity was however unchanged in both groups, indicating increased anxiety in treated rats. To keep in mind is that the groups were small.

Cylinder Test

The rats were put inside a high glass cylinder and their movements were recorded for 10 minutes. Each time one of the paws, left or right, was used against the glass it was counted. However, the cylinder was quite small in radius which gave the rats some trouble moving around and whichever side the glass was of the rat, that paw would be used one or more additional times than the opposite when trying to climb the glass wall. This was done on both sides   which   should   minimise   the   error   margin.
The rats in this study showed no preference to neither left nor right paw, indicating no decrease in motor functions due to treatment.

Social Interaction

In this test, two rats were tested and recorded at the same time which resulted in the two cages being close to each other in order to both be captured on video. This sometimes made the testing rats curious of each other instead of the intruding rat. Two rats would on occasion start fighting loudly which would steal the attention of the neighbouring rats. The cages used were also small which forced the rats to interact more than when a bigger cage was used. Treated rats showed less interaction time compared to control, however not significant which would further indicate slight anxiety in treated rats.

Y-maze

The Y-maze is used to test out memory and spatial learning, which hippocampus governs. The maze was elevated in order to prohibit the rats from escaping and forcing them to explore the arms, however, the walls were not high enough to prohibit the rats from looking outside. The elevation might have served as additional drive for the rats to explore the arms. The rats showed no difference in spatial and memory learning indicating the treatment not damaging hippocampus.

Sugar Preference

The rats showed a clear preference to sugar water and no significant difference was found. This indicates that the rats show no signs of anhedonia.

Inflammatory markers

The cell count for Iba1 was incomplete as some of the rats did not have enough slices of the hippocampus mounted. This ended in some of the rats having only one or two counted series to make an average on. This will make the t-test less reliable, but what can be seen in the tests is that there is a tendency towards less inflammation in the hippocampal region of the treated rats. This could be due to the antibody used is a IGg1, which facilitates an immune response by attracting macrophages. Iba1 is a protein upregulated on activated microglia and macrophages. If the antibody facilitates the immune response, making the process faster, the activated microglia would recede faster and less would be found on stains.

Conclusion

The larger aim of this project is to investigate whether a change in N-cadherin may alter the epileptogenesis and perhaps lower the seizure frequency. The first pilot study of the project was to assess the method by, *in vivo* use of an antibody. The first step was to find a working concentration which can be detected and measured without any adverse effects. In this regard, the result of having 1000µg/ml antibody might be in huge excess, this is however the only concentration at which we have been able to detect the infused antibody. If this concentration works or not, or is even too high, is to be further assessed. As of now, 1000µg/ml does not appear to have any dire outcome and will be used as treatment concentrations. Furthermore, a second pilot study is now ongoing to try and increase the spread of the antibody in the hippocampus by evaluating different cannula coordinates. The final step is the assessing of the synergistic effects with Keppra and N-cadherin antibody treatment will however not be performed by me due to lack of time. The project is ongoing and in the future assessments such as investigating what biological function the loss of N-cadherin at 4 weeks post SE has.

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POPULÄRVETENSKAPLIG SAMMANFATTNING

Hur påverkar klisterprotein epileptiska anfall?

Hjärnan är ett fascinerande komplext organ som styr kroppen. För att hjärnan ska kunna skicka signaler använder hjärnan sig utav små budbärare. Dessa budbärare springer från en kontaktpunkt till en annan mellan nervceller och därmed vidarebefordrar meddelandet. Området där de springer mellan nervcellerna kallas för synaptiska klyftor, där vissa proteiner har som uppgift att klistra fast sig mot varandra från ena cellen till den andra för att inte klyftan ska bli för stor så att budbärarna går vilse. Ett av dessa klisterproteiner är N-cadherin.

Vid sjukdomen epilepsi får man anfall som härstammar från att hjärnan börjar skicka signaler synkroniserat i hela eller en del av hjärnan. Dessa anfall kan uttrycka sig som allt från obehärskade muskelspasmer till små tidsperioder av mental frånvaro. Idag finns det medicin mot dessa anfall, där de flesta medicin påverkar budbärarna genom att till exempel göra så att budbärarna inte kan lika lätt skicka sitt budskap vidare och därmed göra det svårare för hjärnan att synkronisera signalerna. Denna medicin fungerar däremot inte på alla och en stor del av alla med epilepsi får ingen hjälp alls.

Tidigare studier har visat att råttor som fått epilepsi men inte utvecklat återkommande spontana anfall har efter fyra veckors tid mindre av klisterproteiner N-cadherin i hjärnan. Detta tror man har hänt som en försvarsmekanism av hjärnan för att minska anfallen. Man tror att om det finns mindre av klisterproteinet som binder nervcellerna till varandra får man också en större synaptisk klyfta där budbärarna springer. Om detta område blir större blir även budbärarna mindre effektiva på att skicka sina meddelanden vidare och därmed motverkas synkroniseringen.

För att studera detta fenomen har man använt så kallade antikroppar i råttor, en av kroppens egna försvarsmolekyler som fäster sig på specifika platser på proteiner och i denna studie har man använt en antikropp som fäster sig på N-cadherin. Antikroppar främjar kroppens egna försvar och kallar på så kallade makrofager som är kroppens egna underhållsarbetare som arbetar genom att äta upp allt som inte ska finnas där. Genom dessa antikroppar hoppas man på att kunna få bort en del av klisterproteinerna för att återskapa fenomenet man sett hos råttorna.

Om detta ska kunna användas som framtida medicin måste man se en effekt hos råttorna och denna effekt ska innehålla så få biverkningar som möjligt, därför används en del beteende tester på råttorna. Hittills har försöken visat att råttorna som fått behandlingen mår generellt sätt bra men visar en liten trend på att vara ängsliga. Mer försök kommer att göras bland annat för att se hur behandlingen påverkar anfallen, även i kombination med ett redan tillgängligt läkemedel mot anfall. Förhoppningsvis kommer denna studie visa positiva resultat för att man därmed ska kunna forska vidare och skapa en helt ny sorts behandling mot epilepsi.