UiT The Arctic University of Norway

Department of clinical medicine

The effect of one-hour hypothermia on long term spatial memory in male Long Evans rats

Håvar Marsteen

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Supervisor

Kirsten Brun Kjelstrup — Department of Clinical Medicine, UiT the Arctic University of Norway

Co-supervisor

Vegard Heimly Brun — Department of Clinical Medicine, UiT the Arctic University of Norway

Foreword

We have in this paper sought to understand if one hour of general hypothermia can affect the long-term spatial memory in male Long Evans rats. The study was conducted at the Neurobiology research group at the University of Tromsø-The Arctic University of Norway (UiT) under the supervision of Kirsten Brun Kjelstrup and Vegard Heimly Brun in cooperation with the Anesthesia and Critical Care research group also at UiT.

This paper is the culmination of four years of research done as part of the medical student research program at The University of Tromsø - The Arctic University of Norway. The first six months were used to conduct the experiments. The next four and a half years were used to analyze data, read literature and focusing on the medical studies. It is safe to say that things did not go as planned. The closing of the university's animal facility happened just six months into the project. Had we not started the research the proceeding summer with a summer grant, we would probably not have had any data at all. But regardless of this I have been allowed a glimpse into how knowledge is created, how much we know about the brain and how much there is left to discover. There have been ups and downs and I have gained valuable insight into how fickle science really is. But most important of all, I have not been deterred from a career in research.

Acknowledgments

This paper would not have been possible without the help of my supervisors, Kirsten and Vegard and the other participants of our research group, Kamilla Gjerland Haugland, Lorenzo Ragazzi and Angel Moldes. I would like to thank Timofey Kondratyev, Brage Håheim and Torkjel Tveita from the Anesthesia and Critical Care research group for letting us use and their equipment for cooling the rats. I would like to thank Kristian Lensjø, Marianne Hafting Fyhn and Torkel Hafting from the Hafting-Fyhn Neuroplasticity group at the University of Oslo for all help with the lesioning procedures, and for allowing me to stay and feel like part of their group. I would like to thank my parents, my sister and my friends for all their support. And lastly, I would like to thank my girlfriend Fanny Stette for her support, love and help during these studies.

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Abstract

The human body is willing to expend great amount of energy on mechanisms that keep the core temperature within a very small range. When these mechanisms are overwhelmed the body temperature either rises (hyperthermia) or drops (hypothermia). Hypothermia has earlier been used to induce retrograde amnesia (forgetting) and we have in this study examined if hypothermia can affect a robust long-term spatial memory like that of the water maze task.

Thirty-three male Long Evans rats were trained in the water maze task with eight sessions per day for five consecutive days. On day six a baseline Atlantis test was run before randomly allocating the animals to either deep hypothermia (24 °C), intermediate hypothermia (26, 28, 30, 32 °C) and normothermia (37.5 °C) procedure. The target temperature for the deep and intermediate hypothermia was sustained for an hour and the normothermia time controlled to the mean time spent under anesthesia for the hypothermic groups. The animals then rested for five days before a final retrieval Atlantis trial was conducted.

We found no difference between the three groups during training, at baseline or at retrieval. This meant that there was no difference in how well the groups learned the task and there was no difference in how well the groups remembered the task after five days. We did not find any FJC positive cells in the histology, but due to the lack of positive controls we cannot draw any conclusions from this.

The results from this study seem to suggest that hypothermia do not induce retrograde amnesia for the water maze task, compared to normothermia. A confounding factor might be the noise resulting from the construction work going on just outside the animal facility and the low number of animals in the study.

1 Introduction

Mammals, including humans, are dependent on internal homeostatic mechanisms in order to keep the body temperature at 37 degrees Celsius (°C) for biological function. Outside the optimal temperature range, cellular processes are impaired and ultimately lead to multiple organ failure and death.

The last two decades has provided case reports of survival from accidental hypothermia, with the lowest body temperature recorded at 13.7 °C (1) only recently beaten by a Polish two year old surviving a core temperature of a mere 11.8 °C (2). Similar feats of survival inspired the medical profession to use controlled lowering of the body temperature in a therapeutic, or protective, manner when the brain´s oxygen supply is threatened, from as early as the 1950's. Thus, deep hypothermic circulatory arrest became the key component allowing the surgeons to operate on the aortic arch (3). Another aspect is the fact that children suffering from hypoxia during birth show enhanced survival in asphyxiated newborns (4). On the other hand, preemptive hypothermia has been applied to patients surviving cardiac arrest (5-7) and traumatic brain injury (7, 8) , with no effect on outcome.

Research into how the body is affected by hypothermia is a varied field with a lot of ongoing research (9). But the effect of hypothermia on the normal brain is not completely understood. We have in this study examined if one hour of hypothermia can affect the long-term spatial memory in male Long Evans rats compared to normothermia.

1.1 Hypothermia

Hypothermia is in humans defined as a core temperature (T_c) below 35 ℃. As T_c decreases, the symptoms increase. They span from light shivering and a feeling of coldness, all the way to severe hypothermia including disorientation and cardiac arrest (10-12).

1.1.1 Stages

1.1.1.1 Mild (I)

The mildest symptoms occur between 35 and 32 ℃, defined as mild hypothermia. These symptoms include shivering, fatigue and nausea. Some mild cognitive symptoms may be present, among others impaired memory and judgement (10-12).

1.1.1.2 Moderate (II)

When the core temperature drops to between 28 and 32 ℃, we reach the moderate hypothermia state. In this state, the hypothermia is starting to affect the brain and heart. The cognitive capacity is severely reduced and now the hyporeflexia might set in along with the lethargy. The shivering is often nonexistent at this point and the risk of cardiac dysrhythmias is increasing (10-12).

1.1.1.3 Severe (III)

Core temperature between 28 and 24 ℃ is denoted as severe hypothermia. The patient is often unresponsive and all vital signs continue to decrease. The blood is being centralized and water excreted through the kidneys, leading to dehydration. This, in addition to cardiac dysrhythmia increases the risk of ischemia to the brain (10-12).

1.1.1.4 The later stages (IV+ V)

The Swiss Staging system uses two more stages where stage IV (T_c< 24 ℃) is associated with cardiac arrest and stage V (T_c< 13.7 ℃) is the lowest recorded $T_c$with following recovery (1, 11). These stages are not relevant for this study and are not directly transferable to the rat model, and thus will not be discussed further.

1.1.2 Accidental vs therapeutic hypothermia

Hypothermia in human patients is categorized as accidental or therapeutic. The accidental hypothermia describes all situations where the hypothermia is unintentional, for example accidental cold water drowning or during anesthesia in a badly heated surgery room. The

therapeutic hypothermia describes all situations where the hypothermia is induced intentionally. The use of therapeutic hypothermia has allowed huge leaps in fields like surgery and emergency medicine. Within surgery it has allowed the transport of transplant organs over greater distances, and within emergency medicine temperature control now is an important factor in the treatment of stroke and cardiac arrest (10, 11). Although therapeutic hypothermia is used in clinical practice, we still know very little about how and what pathways in the body hypothermia affects. Only the accidental and therapeutic hypothermia in a surgery/laboratory setting in smaller lab animals will be in the scope of this study.

1.1.3 Heat loss

For hypothermia to occur there must be a net loss of heat to the environment. The heat is lost through four main processes; radiation, conduction, convection and evaporation. The heat loss through radiation is the most significant, accounting for approximately 60% of all lost heat. Almost all radiation from the human body radiates as photons within the wavelength spectrum of infrared light. Since infrared wavelengths are easily blocked by clothes or other insulating fabrics, this is an effective way of insulating. The conduction of heat is dependent on contact and is the direct movement of kinetic energy between molecules at the atomic scale. Transfer of heat through a combination of conduction and radiation will in turn heat the gas or fluid close to the body of heat, in most instances this will create turbulence thus leading to a convective heat loss as new cold fluid or gas substitutes the newly warmed. Evaporation is the last mechanism of heat transfer and occurs when a phase change is initiated, this phase change is an endogenous process requiring energy. It is important to note that transfer of heat is viewed as a net process. If the heat transfer from the environment to the body is greater than that from the body to the environment, we do not loose heat but gain it. Thus, the temperature gradient is an important factor of heat loss (10, 12, 13). The medium the heat is transferred through is also an important factor. Water has a heat transfer capacity much higher than air and thus transfer heat much quicker.

1.1.4 Thermoregulation in the mammalian body

Thermoregulation in mammals consists of a connection between peripheral cold sensors (mostly in the skin) and the brain. As the external temperature decreases, the body increases

its own heat production and limits its heat losses mainly through behavioral changes and centralization of the blood. By doing this, the body can maintain a higher core temperature for longer despite the cold environment (10, 12).

1.1.4.1 Behavioral changes

The arguably most effective defense against heat loss is the behavioral changes associated with feeling cold. As the temperature of our surroundings decline, we immediately feel the urge to get warmer. This can be achieved through increased activity, increasing insulation or by moving to a warmer environment. By changing the environment, the body expends little energy in relation to the heat gained (10).

1.1.4.2 Centralization

During hypothermia, the body will protect important organs like the brain, heart and lungs etc. This is done through constriction of the peripheral blood vessels, effectively moving the blood to the thorax. The torso is the main hub of important organs and also the most voluminous part of the body. Due to its shape, the surface area to volume ratio will be the smallest in the thorax, thus minimizing heat loss (10, 12).

1.1.4.3 Shivering

Another mode of defense is the well-known shivering. By contracting muscles and burning sugar and fatty acids heat is produced in the conversion of molecular energy into movement (10, 12).

1.1.4.4 Centralization and shivering as contradictive processes

The shivering and centralization are contradictive processes in the sense that they are dependent on being balanced against each other. This is because many muscles are located in the extremities. The distal vasoconstriction slows much of the blood flow to and from the extremities. Thus, the deliverance and removal of substrate to the muscles are lessened and less productive. At the same time, the heat produced will be less efficiently transferred to the

central organs. This is of course a balanced process and the heat generated in the peripheral muscles maintains function and keeps them ready for use.

1.1.4.5 Non shivering thermogenesis

Brown adipose tissue converts lipids and carbohydrates into energy in the form of heat. It produces no usable work, has a lower isolation factor than beige adipose tissue and is closely related to muscle tissue. The BAT cells have numerous mitochondria which allows the proton pump in the Electron transport chain to create heat instead of water. This process is called non shivering thermogenesis. The BAT cells are rare in the adult human, but newborn and hibernating mammals has high levels of BAT. Rats can, because of BATs and other compensative mechanisms, be acclimatized to live in cold environments (14).

1.1.5 Anesthesia and hypothermia

Many anesthetic drugs shift the body's own threshold for thermoregulation away from the point of neutrality, while also stopping some of the processes activated to counteract the shift. An example is isoflurane which dilates the peripheral vessels, thus counteracting the centralization of the blood. While the temperature needed before compensatory mechanisms is activated in lowered/heightened. Hence, the bodies thermoregulation is severely diminished. This explains the passive hypothermia often seen during surgery (14).

1.1.6 Neuroprotection of hypothermia

Neuroprotection is the ability to limit sustained damage to neurons or the brain. In the laboratory setting hypothermia has shown itself to be one of the most effective neuroprotectants so far (15). For instance, in the case of cerebral ischemia it has been demonstrated that hypothermia decreases the metabolism of the brain cells in piglets at a rate of approximately 5% per degree (16). Also the spreading damage of excitotoxicity in the acute phase, the programmed cell death (apoptosis) and inhibition of inflammation in the subacute phase and lastly enhanced recovery in the chronic phase are affected in a positive manner through hypothermic treatment (15).

1.2 Neurobiology

Neurobiology is the branch of neuroscience working on explaining what we see in behavior, into circuits of cells and cellular mechanisms. By describing and understanding how cells act and interact to create behavior, our understanding of how to manipulate and treat various illnesses affecting the brain increases.

1.2.1 Hippocampus

The hippocampus is an important and easily located brain area. Located in the medial part of the temporal lobe, the visual similarity to the seahorse has led to the name, which is Latin for seahorse. The hippocampus became the very center of research into memory in 1957 when Scoville and Millner published a case report regarding patient H.M., whom had undergone an experimental procedure to alleviate his temporal epilepsy. By attempting to remove the foci of the seizures located in the medial structures of the left temporal lobe, which included the anterior three thirds of the hippocampus. As a result, H.M. was later unable to form new memories, although he retained autobiographical memories from before the surgery. This suggests that the parts removed was an integral part of the memory forming structures, but not an integral part of the circuits responsible for storage and recollection of memories (17).

1.2.1.1 Long term memory

The creation of a long-term memory consists of two distinct phases, acquisition and consolidation. The acquisition phase, if simplified, is the process of creating a short-term memory which later can be consolidated into a long-term memory. The hippocampus poses an integral role in the consolidation process, which is in line with the finding by Scoville and Milner. The time frame of this initial consolidation process in not known, but it varies from a few hours to a few days. This is because the movement of the memory to long term storage in the cortex of the brain is dependent on protein synthesis and gene expression as well as synaptic plasticity (18). Sleep is also a very important part of the consolidation process, specifically the slow wave sleep proceeding REM sleep. The stabilization of the newly consolidated memory can continue for months or even years (19-21).

When recollecting an episode, the memory becomes susceptible to change before being consolidated again. For a long time, the leading theory was that the old memory would then be deleted and the new memory take its place. Newer research suggests that the old memory is not deleted, but rather that a whole new memory is stored. This process is called reconsolidation. It is believed that retrograde amnesia, the act of forgetting, is a disruption of the reconsolidation process, thus disrupting the new memory (19-21). In anterograde amnesia, the patient is not able to form new memories. This occurs when the original consolidation process is disrupted, as in the case of patient H.M(17).

1.2.2 Hypothermia and amnesia

Research into hypothermia induced amnesia done by Riccio, among others, found that exposure to hypothermia following a single trial passive aversion task induced amnesia for the task (22-24). The experimental setup consisting of a two-compartment box, one black and one white, separated by a guillotine door and with metal railing floor. The rat was put in the white compartment and after moving over to the black, the door was closed and the rat received an inescapable shock. As a result the rat learned to avoid the black compartment. Next the animal would be cooled by submersion in cold water while restrained either 20 sec, 5 min or 15 min after the task. Then the day after, the same task would be presented and the latency for moving from the white to the black compartment was recorded. Riccio found that the retrograde amnesia was dependent on the time between the task and the hypothermic exposure, the greatest effect was seen in the 20 sec group (23).

1.2.3 Spatial navigation

The hippocampal formations special role in navigation became clearer when O'Keefe and Dostrovsky discovered the hippocampal place cells. During their research, using signal probes lowered into the hippocampus, they discovered cells that only fired in specific locations in an environment. They then postulated that these specific cells were an integral part of mapping out an environment and most importantly for noticing changes in that environment (25). Later O'Keefe and Nadel proposed in their literary review that the role of the hippocampus was to create cognitive maps, linking positions in space (26).

1.2.4 Declarative memory

A recent view on the memory formation in the hippocampus is called the declarative memory function of the hippocampus. According to this theory, the lack of memory formation in the lesioned subjects is attributed to the inability to create associations between objects. Arguing that the hippocampal formation not only supplies the brain with a spatial mapping, but a temporal and dimensionless memory space where the links between the sensory input not only is stored in a sequential form (like a film roll) but in the form of similarities tying them together to create a memory. This way of thinking about memory formation gives the hippocampus a much more organizational role in the creation of memories. Directing and sorting the flow of sensory input into a form of organized memories. Without this organization, no new memory can be formed (27, 28).

1.2.5 Spatial memory and the Morris water maze

Spatial memory is the ability to remember relations in space. This holds true for smaller rooms, but also for bigger open spaces. The fact that you can remember where you parked your car in a parking lot, and locate your pen in the office, is a testament to this. A much-used test for spatial memory is the water maze, initially described by R. G. M. Morris (29). The water maze is a large circular pool filled with opaque water and has a hidden platform submerged just below the surface. The research animal swims until it finds the platform. Using visual cues around the pool to navigate, the animal can learn the spatial location in relation to the visual cues. An analogy to the task is fishermen who navigate using constellations or landmarks to guide them to the fishing grounds.

2 Methods

2.1 Ethical statement

The use of animals in this study was done in accordance with the Norwegian Regulation concerning the use of animals for scientific purposes and was approved by The Norwegian Food Safety Authority through the FOTS Application id 8169.

2.2 Study design

To understand the effect of hypothermia on long term spatial memory, we conducted a study with three groups, deep hypothermia, at 24 °C, intermediate hypothermia at 26-32°C, and normothermia at 37°C. A non-intervention group was also used, but later included into the normothermia group. The animals received five days of training in the Morris water maze. On day six, the platform was made unavailable while the animal was searching. The search pattern during this Atlantis test provided a baseline level of the memory. After the Atlantis test, the deep hypothermia, intermediate hypothermia and the normothermia group where subjected to intervention, while the non-intervention group where kept in the housing room as a control. The animals recovered for four days before the final retrieval test was conducted as an Atlantis test. Immediately after the retrieval test, the animals were sacrificed and perfused, and the brain extracted for histology.

2.3 Experimental animals

The animals used in the study consisted of 23 male Long Evans black hooded rats (Charles River, Italy). On arrival, all rats were six weeks old and were given a quarantine period of one week. At this point, the animals waited in their cages. At inclusion to the experiment they went through up to three days of handling before experiment start. The age at start of training in the water maze ranged from seven to fourteen weeks. The rats were weighed each day during the experimental period. The mean starting weight was 361 g and the mean weight at euthanasia were 380 g.

In addition, two rats (Male Long Evans, Charles River, Italy) where included in the spring of 2020 to serve as histological controls with detectable brain damage. These rats were housed at the animal facility at the biology department at the University of Oslo.

2.4 Housing

The rats were housed two or three together, in a clear plastic bottomed and metal grating covered cage. Their environment was enriched by having sawdust as the flooring, and shredded cardboard as nesting material. They also received a red plastic tube to play with.

The room was set to a 12h inverted night/day cycle, lights on at 7 pm. The humidity and temperature were controlled, humidity (55 ± 5%rh), temperature (21 ± 1°C). The animals had free access to Food and water except for 48 hours following intervention, during which the animals would be housed in separate cages with free access to water and 20 food pellets, refilled two times pr day, to monitor food intake.

2.5 Humane endpoints

For the experimental period, the humane endpoints were weight loss >10%, scored stress and Neurologic deficit score (NDS). During procedure, endpoints were cessation of ventilation or a massive bleed. If any animals reached a humane endpoint, the animal would be excluded from the study and euthanized.

2.5.1 Stress score

The animals stress level was scored once every day through the experiment. The score was based on respiration frequency, weight loss, food intake, fur quality, NDS and social behavior. Normal value/behavior was scored with a value of one, close to normal with a value of two and pathological with a value of three. Maximum score was 20, while a normal score was seven. If any animal reached a score of 10 or higher the animal would be euthanized immediately. A score between seven and ten would require veterinary assessment of the health of the animal. See appendix 1.

2.5.2 Neurologic deficit score

The neurological function of each animal was assessed at least two times during the training period and once daily during recovery after the procedure. The scoring was based on an assessment of general behavioral deficit, brain stem function, motor assessment, sensory assessment, motor function, social behavior and if there were seizures present. The general behavior was assessed based on consciousness, spontaneous arousal and respiration. The brainstem function was scored based on olfaction, vision, pupillary light reflex. Corneal reflex, hearing, whisker stimulation reflex and the ability to swallow liquids and solids. The motor assessment was assessed based on strength of right side and left side. Sensory

assessment was assessed based on pain withdrawal of right and left side extremity. Motor function was assessed based on gait coordination, balance on beam and righting reflex. The social behavior was assessed based on spontaneous exploration and observed signs of anxiety. A normal score was set to 86 points. See appendix 2.

2.6 Layout of the experiment room

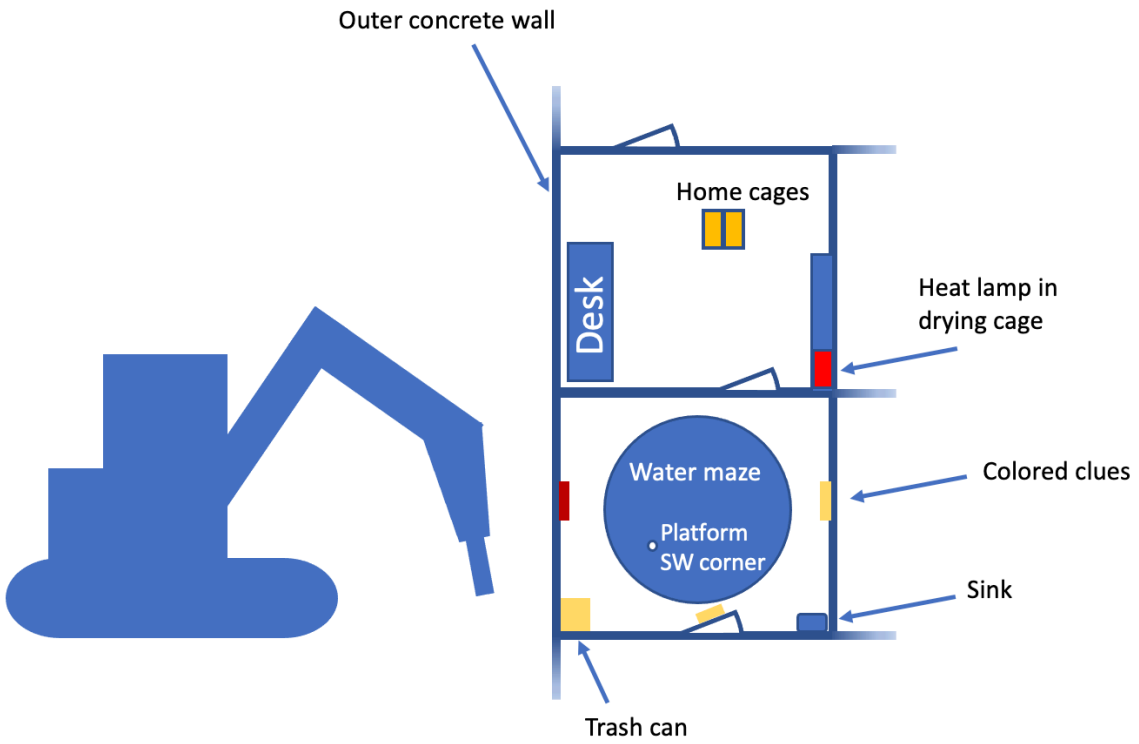


Figure 1 Experiment room layout. Here the layout of the room used for the water maze training and testing is depicted. The experimental rooms were located at the end of the animal facility and had an outer concrete wall. From the summer of 2016 there was construction work going on outside the wall.

Figure 1 depicts the layout of the experimental rooms used for the water maze training and testing. The room with the water maze was approximately ten square meters, with yellow walls, a sink approximately 50 cm off the floor. In the south west corner of the water maze room there was a yellow trash can that was not visible from the surface of the pool. All four walls had color cues, yellow a4 sized square on the east wall, a red on the west, a yellow on a

blue door to the south and a plane blue door to the north. The next room to the north was the operator room, where the operator sat with the computer. The drying cage was also located in this room, as well as the storage of the other rats. The door between the rooms could not be closed due to the camera cable running through it. The experimental rooms were located at the end of the animal facility and the wall to the west was an outer wall made from concrete. Starting the summer of 2016 there was construction work going on outside the wall and increasing through the year. The noise and vibrations this created was impossible to avoid due to construction work both during the day and the evenings.

2.7 Experimental procedures

2.7.1 Water maze

The water maze (see figure 1) used was a white custom-made fiberglass pool, 2m in radius, 50cm high. The transparent platform (On-demand water maze platform for mouse, Med Associates, Inc, Fairfax. Virginia, USA) used an electronic pneumatic system for lowering and raising, controlled by a manual switch in the next room. The platform was located in the South West (SW) quadrant for all tasks, but there were three other potential platform positions North East (NE), North West (NW) and South East (SE). The visual cues were as described in the paragraph above. The pool was filled up to two cm above the transparent platform when raised. White opaque water was made by adding 80 ml of white nontoxic watercolor to the pool after it was filled and kept at 21-23 °C for all experiments. The white water was used to contrast the black hood of the Long Evans rats, allowing tracking via a camera (Axona Ltd, St.Albans, U.K) mounted to the roof and centered above the pool, tracking software (Dacq acquisition system, Axona Ltd, St.Albans, U.K) was used to track the animals positions in the pool.

2.7.2 Water maze procedure

2.7.2.1 Training protocol overview

In order for the animals to learn the water maze task a training regime consisting of four trials per session and two sessions per day for five days were conducted. Between each session, the animals were allowed a minimum of two hours to rest. The first trial of the first session of each day was an Atlantis test.

Each trial started with the animal being released from one of the four starting positions (North, West, South or East). The starting position for each trial were randomized at the start of the study and all animals received the same order of starting positions. The trials lasted a maximum of 120 seconds and was ended when the animal found the platform, or the 120 seconds expired. If the animal found the platform, it was allowed to rest there for 30 seconds before the next trial was started. If the animal was unable to find the platform before the 120 seconds had passed, it was led to the platform and allowed 30 seconds of rest. Drying was done under a 175-watt heating lamp (Felleskjøpet, Tromsø) in a fresh plexiglas cage with a red tube and a white folded towel for 20 minutes. After drying, the animal was put back in its home cage.

2.7.2.2 Atlantis test

To quantify the animal´s memory of the water maze we used an Atlantis test. In this task, the animal was released from a random location (North or East) and the platform was lowered out of reach of the animal for the first 60 seconds of the trial. Because there was no platform, the animal was forced to search for the location where it remembered the platform being. After the 60 seconds had passed the platform was raised. The animal was then given another 60 seconds to find the platform. If the animal did not find the platform it was led to the it and allowed 30 seconds of rest before being dried according to the procedure described for the training trials above.

2.7.2.3 Overview of water maze regime

For the first five days, the animals were trained according to the training protocol described above. In order to have data on the learning progression of the animals the first trail of each training day was an Atlantis task. On day six a baseline for memory was set using an Atlantis task. After drying, the rats were transferred to the lab and randomly allotted to either hypothermia or normothermia procedures. After four days of rest a retrieval Atlantis test was conducted, and the animals euthanized.

2.7.3 Analysis of the water maze trials

To quantify the performance in the water maze trials we measured the time from release to either finding the platform in the training trials or to the first pass of the platform position in the Atlantis test. This is the latency of the animal to find the platform. For the Atlantis trials we used the roof mounted camera and DacqTrackWM software to track the animals swimming paths. These paths consisted of the position of the animal recorded 25 times a second given as x- and y-coordinates. We then calculated the time spent within the target zone, set to 30 cm outside the platform, and the mean cumulated distance to the platform. Both calculations were conducted using a custom MATLAB script written by Vegard Brun and reworked by Håvar Marsteen.

2.7.4 Anesthesia

To induce anesthesia, the animal was taken directly from drying after the baseline Atlantis test on day six, and put in a clear induction chamber saturated with 5% isoflurane gas from a vaporizer (Univentor 410 Anesthesia unit, Zejtun, Malta) driven by room air from a small aquarium pump. When the animal stopped moving it was put in prone position on a water circulated aluminum tabletop. From this point on, continuous anesthesia was administered through a mouthpiece and the isoflurane (saturation and flow) was titrated based on respiratory rate, oxygen saturation and lack of pinch reflex.

Analgesics was then administered in the form of 0.5 mg/kg buprenorphine (Temgesic) and 2.00 mg/kg meloxicam (Metacam) injected subcutaneously in the neck and given 10 minutes to take effect. To avoid the eyes drying out, Simplex salve (Tubilux Pharma S.p.A, Pomezia, Italia) was administered directly to both eyeballs. A water-circulated probe was inserted in a thermometer sleeve and the sleeve lubricated using ultrasound gel before being inserted in the rectum of the animal. To record core temperature a probe (Thermalert Th-5 thermocouple controller, Bailey Instruments, England) was inserted in the esophagus and checked to ensure it did not interfere with breathing. An infrared tab placed on the right paw (Physiosuite, Kent Scientific, Torrington, USA) measured heartrate, saturation and respiratory rate. The values were recorded manually every fifth minute. After emergence from anesthesia, the animal was put in a clean cage and observed until awake and moving. The animal was for the next 48

hours housed individually. During this time, it was weighed two times a day and monitored for food intake. It was also monitored for stress (appendix 1) and neurological deficits (appendix 2) and administered analgesics in the form of 0.05 mg/kg buprenorphine every twelve hours and 2.00 mg/kg meloxicam every twenty-four hour.

2.7.5 Hypothermia protocol

To induce hypothermia, the circulating water temperature of the tabletop and the rectal probe was set to 10°C. The water temperature was constantly adjusted in relation to the animal´s core temperature to avoid overshoot. As the animal´s core temperature became lower, the breathing slowed and thus the amount of anesthetic also had to be titrated. Signs of too shallow anesthesia, as shivering or posture change, were monitored as well as those listed for the anesthesia protocol. The animal was kept at target temperature for an hour before being rewarmed by increasing the target temperature of the circulated water to 37.5 °C. The rate of rewarming was closely monitored and not allowed to exceed 0.25 °C per minute to lower the lethality (30). When the core temperature read 36.5 °C, the procedure was concluded, the animal moved to its cage and allowed to awake.

2.7.6 Normothermia

To control for the effect of the anesthesia on the hypothermic group, a normothermic group, kept at 37 °C, was created. This group were all trained, tested and anesthetized according to the same protocols as the hypothermic group. During anesthesia the temperature was kept at 37 °C and the procedure time was the same as the average for the hypothermia procedures.

2.7.7 Lesioning

To verify that the FJC staining protocol was working a positive control with known damage was needed. Using a Nanoject III syringe (Drummond scientific, USA) and a stereotactic frame (World Precision Instruments, Hertfordshire, United kingdoms), two 9 weeks old male Long Evans rats, was bilaterally injected with ibotenic Acid (Fischer scientific, Geel, Belgium). Due to the animal facility in Tromsø being closed down, Hafting-Fyhn Neuroplasticity group kindly helped us produce the lesioning lesioned animals. The animal

was induced and received analgesics according to the anesthesia protocol described above. They were fastened in the stereotactic frame using ear bars and a bite frame with a nozzle for the isoflurane. When the head was stable, the fur on the top of the head was shaved off. A 10mm incision was done from bregma to lambda and the skin and facie on the crown of the head was loosened and the scull laid bare. Measuring from bregma, the positions for injection was marked and using a diamond burr small holes were drilled through the scull. Through the holes the ibotenic acid was injected at 4µl/h. The quantities and coordinates used are given as µL and µm posterior, lateral and ventral from bregma: 0.15 µL at 4.0 µm, 3.1 µm, 2.9 µm, 0.08 µL at 3.5 µm, 3.4 µm, 2.9 µm and 0.05 µL at 2.8 µm, 3.0 µm, 2.9 µm. The coordinates and quantities were gathered for lesioning of CA3 from the protocol by Jerman et.al, 2005 (31). The animals were sacrificed after two days.

2.8 Perfusion

At the end of the experimental period, the animals were sacrificed. The induction chamber was prefilled with 5% isoflurane, and the animal placed inside it. As the animal stopped moving it was given a subcutaneous injection of 0.5 mg/kg buprenorphine and then an intraperitoneal injection of 100mg/kg of pentobarbital. After the animal had stopped breathing, the animal was taped to a grate above a surgical bowl to collect debris and fluids. The skin and muscle just below the xiphoid process was cut using surgical scissors exposing the diaphragm which was cut and the chest cavity opened by cutting the ribs along either side of the chest. Using forceps to hold the xiphoid process, the sternum was bent up. By using a peristaltic pump and a 23G needle inserted into the left ventricle, the animals were perfused transcardially after cutting open the right atrium. First with 1x phosphate buffered saline (PBS) until clear liquid drained from the right atrium and then with 4% paraformaldehyde in PBS. The brains were then extracted and stored in formaldehyde for 48 hours before it was moved to a Dimethyl sulfoxide (DMSO) solution and stored in a fridge at 4 °C until processing.

2.9 Histology

To cut the brains a Leica M1950 Cryostat (Buffalo Grove, USA) was used. The cryostat chamber temperature was set to minus 20°C and the shoe was put on a quick freeze plate set

to minus 50°C. The brains were removed from the storage medium and dried on a clean piece of tissue paper. Using a scalpel, the cerebellum and two millimeters of the frontal lobe were removed. Freezing glue (Color less Neg-50, Ricgard-Allan Scientific) was put on the cutting shoe and the brain placed with the flat frontal lobe down on the shoe. More glue was then administered around the lower part of the brain to stabilize it for slicing. Cold spray was used to quickly freeze the brain and the freezing glue. This was done to minimize freezing artifacts in the brains resulting from too slow cooling. The shoe with the brain was put back on the quick freeze plate and left there for 30 minutes. The brains were then cut in 40 μm sections through the whole brain, at every 10th slice the first and second slice was mounted on Super frost plus slides and dried in the dark for 24 hours. They were then moved to the freezer for storage until the time when they would be stained. The third and fourth slices were stored in DMSO solution as backup.

2.9.1 Fluoro Jade C

This protocol was adapted from Schmued et al (2005)(32).

2.9.1.1 Solutions

From a 30 mg Fluoro jade C (FJC) stock powder (Millipore AS, Oslo, Norway), 5.7mg was extracted and dissolved in 57ml distilled water. Resulting in a stock solution of 0.01%, which was shielded from light, stored at 2-8 °C and used within three months. From the stock solution, 1ml was mixed with 99ml 0.1% acetic acid solution to create 100ml fresh 0.0001% working FJC solution. The working solution was used within an hour and then discarded.

The Potassium permanganate ($KMnO^4$) solution consisted of 150mg $KMnO^4$ dissolved in 250ml distilled water. Resulting in a 0.06% solution, that was shielded from light.

2.9.1.2 Staining

For the staining procedure 12 slides, containing four slices each were loaded in a carriage. The carriage was first submerged for five minutes in a solution of 80% ethanol and 0.01%

NaOH. Then two minutes in 70% ethanol and a rinsing step of two minutes in distilled water. Next the carriage was put into the $KMnO^4$solution and put on a stirring table (VWR Standard Orbital Shaker, Model 1000) on a low setting for 10 min. As the next steps were light sensitive, exposure was kept to a minimum. After another rinsing step for two min in distilled water, the carriage was left for 10 minutes in 0.0001% FJC solution. Lastly, the carriage was rinsed three times for two minutes each in distilled water. Then the water droplets were removed before drying at 50 ºC for five min. After drying, the slides were put in Xylene bath for at least 2 min. One by one, the slides were taken out of the xylene and coverslip with Entellan new (Merck, Darmstadt, Germany).

2.9.2 Cresyl violet staining

2.9.2.1 Ethanol based solutions

For the staining procedure 50%, 70%, 80%, 90%, 96% ethanol baths were produced by mixing 96% ethanol with distilled water. From the 70% ethanol 500 ml was then mixed with 2.5 ml Acetic Acid resulting in approximately 0.5% acetic acid in 70% ethanol.

2.9.2.2 Cresyl violet solution

The staining solution was created by dissolving 0.5g of cresyl violet acetate (Sigma-Aldrich, Oslo, Norway) in 500 ml distilled water. The solution was then left in the dark on a stirrer at 60°C for three hours. The solution was then filtrated into a non-transparent bottle and stored in the dark at room temperature.

2.9.2.3 Staining setup

The staining setup consisted of 13 units. Seven ethanol baths, one water bath, one cresyl violet bath, one 0.5% acetic acid in 70% ethanol bath and three xylene baths. The cresyl violet bath was put to the left, then the four ethanol baths in increasing concentration with three 96% ethanol baths besides it and a xylene bath at the end. The row below consisted of the 0.5% acetic acid in 70% ethanol bath, a cold distilled water bath and lastly two xylene baths.

2.9.2.4 Staining procedure

The carriage containing 12 slides was dipped 10 times in each of the seven ethanol solutions before sitting two minutes in the xylene bath for clearing. To rehydrate the slices, the carriage was then dipped 10 times in the seven ethanol solutions, but now in descending order and washed quickly under running tap water. After this the carriage was placed in the cresyl violet solution for 6-8 minutes based on the age of the solution and then washed under running tap water. When all excess color was removed the carriage was placed in the ethanol with acetic acid solution for a few seconds while gently shaking the container then quickly moved to the cold-water bath until all excess color was washed away. The ethanol with acetic acid and cold-water bath step was repeated until the sections were sufficiently light, while still showing good contrast. Then the slides were dehydrated again with ten dips in increasing concentration of ethanol as in the first steps of the procedure. And in the end moved to the last two xylene baths for clearing. The first xylene bath for two minutes, the second for at least five minutes up to an hour. The slides were then cover slipped using Entellan new.

2.9.3 Analysis

The slides were manually analyzed on a Carl Zeiss Axio zoom V16 fluorescent microscope (Carl Zeiss Microscopy GmbH, Jena, Germany). The settings used were: 4220ms exposure, 80x zoom, a Carl Zeiss philter nr 38 and the light source set to minimum. The pictures were converted from CSV to TIFF using ImageJ (Version 2.1.0, ImageJ.net).

2.10 Programming the analysis tools.

During the analysis of the behavior data we wanted to analyze for both time in zone and cumulated distance to platform. The original program, DacqTrackWM, used for analysis of the recordings only analyzed for time in zone. To analyze for distance to platform Vegard Brun had a MATLAB (R2020a MATWORKS) script that read the .POS files that were created by the DacqTrackWM containing paired x and y coordinates and the .WST files containing the settings used during the recording and the position of the maze and platform. The calculations were done using two dimensional vectorial math. The script then wrote the values directly into Excel (version 16.41, Microsoft). See Appendix 3 for the whole script.

2.10.1 Calculating the mean cumulated distance to platform

The tracking of the animal, via the DacqTrackWM program, recorded the animals coordinates 25 times every second. Then, using vectorial math for calculating the distance between two coordinates in the x-y plane, the distance between the coordinate position of the animals and the platform center were calculated for all positions. By dividing the sum of all the distances by the number of recorded positions we calculated the mean distance between the animal and the platform. This was done for all four possible platform positions.

2.10.2 Calculating time in zone

To calculate the time spent in target zone we used the same distances between the animal position and the platform, from the calculation above. We then counted all values less than the target zone radius, resulting in the time in zone measurement. This was done for all four possible platform positions.

2.10.3 Generating heatmaps

To create the heatmaps shown in figure 5, figure 6, figure 8 and figure 9, the coordinates were fist pooled across the group and then binned to 20 x 20 bins. The bins were then normalized to allow comparison. This was done by dividing the value of each bin by the number of positions in the pooled group data and multiplying it by the length of the trial. The heatmap was printed by adapting a script found on MATLAB answers, by the user Neuropragmatist (33), and using a interpolation shading to smooth out the heatmap.

2.11 Experimental outcomes

To quantify the loss of memory, we used the difference in preference for the platform locations in the Atlantis trial before and after intervention. This way we hoped to answer the research question of if and how hypothermia affects long-term spatial memory. And, by cooling to temperatures 24, 26, 28 and 30, we would be able to determine if the effect has a dose/response property. If there was any detectable effect of hypothermia on long term spatial memory, we also hoped to determine if there was any detectable cell damage in the hippocampus using the fluorescent FJC dye.

2.12 Statistical methods

The positional data recorded from the water maze was processed using a custom MATLAB script. The script wrote the quantitative measurements directly to Excel presorted for rat number and variable. Only the manually recorded latency measurement had to be added manually to Excel before analysis. All statistical analysis was conducted in SPSS (version 26, IBM Corp) with the statistical procedures and assumptions gathered from Laerd Statistics (Laerd Statistics, 2015). All graphs were created using Prizm 8 (version 8.4.3, Graphpad Software LLC) and all figures created using Power point (version 16.41, Microsoft).

For the statistical analysis we used Two-way mixed ANOVA to analyze over the training periods and across the groups. We then used a two-way ANOVA with post hoc analysis to analyze over the platform zones and across the groups at the baseline and retrieval trials. Lastly, we used one-way ANOVA to analyze the mean speed at the baseline and retrieval trials.

2.12.1 Assumptions of the ANOVA

If not otherwise stated, the dataset met the assumptions of normality as assessed by Schapiro Wilks test ($p > 0.05$), lack of outliers as assessed by the boxplot and homogeneity of variance as assessed by Levene's test of homogeneity of variance ($P > 0.05$). The Two way and Two-way mixed ANOVA also met the assumptions of the homogeneity of covariances as assessed by Box's test of covariance matrixes ($P > 0.05$), if not otherwise stated. If outliers were found, they were tested by converting them to their closest value ± 0.01 and stated only if they then affected the result. If the assumption of sphericity was broken, a Greenhouse-Geisser error correction would be applied to the result.

3 Results

We investigated the effect of one-hour hypothermia and anesthesia on long term spatial memory, in male Long Evans rats. For the behavioral experiment, 25 animals were used. After completion of the experiments ten animas from the pilot study, run in 2014, was added to the behavioral material to increase the power of the study. Lastly two animals were used to

produce positive histological controls for use with the FJC staining. In total the study encompasses 35 animals.

The timeline shown in figure 2, shows the chronological inclusion of the animals for each experimental group. The six animals in the deep hypothermia group (blue) were all tested from January to March 2017. Six animals of the intermediate hypothermia group (green) were from the pilot study run in 2014, and the last four animals were trained and tested in July 2016. The normothermia groups (orange) contains four rats from the pilot study, four from July 2016, two from August 2016, two from November 2016 and two from March 2017. In the negative control group (non-intervention), one animal was trained in August 2016 and two more in March 2017. In addition to these animals, as mentioned earlier, two positive histological controls were added in March of 2020.

The two control groups, normothermia and non-intervention, showed no significant difference in any of the behavioral tests (Mann-Whitney – U test for each of the platform positions for the baseline and retrieval Atlantis test all showed $p > 0.05$) and were merged for analysis. .

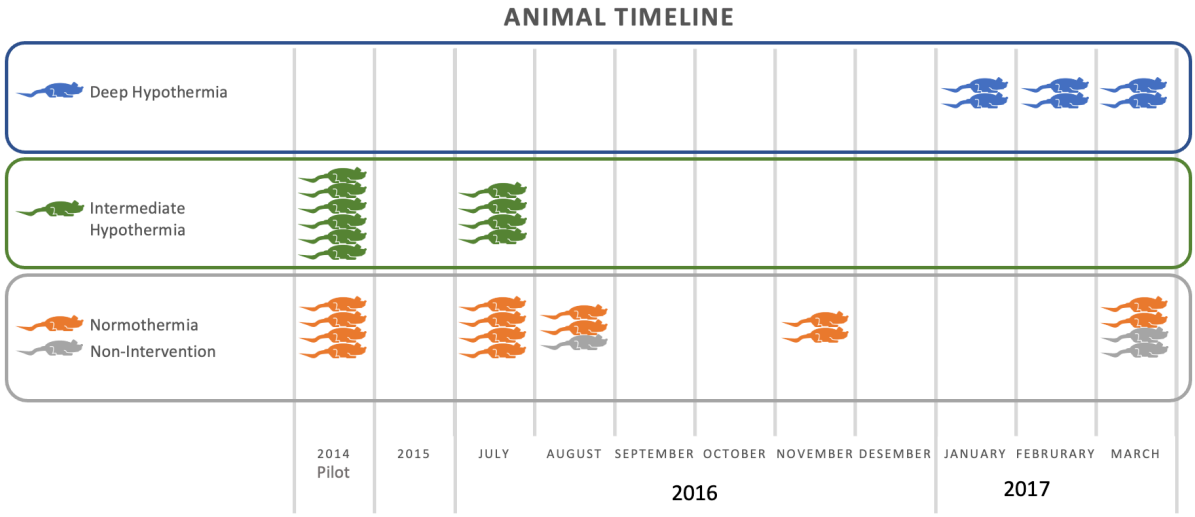


Figure 2 Animal timeline. This is the chronological timeline of the animals for each experimental group. The deep hypothermia group, blue, were all tested from January to March 2017. Six of the intermediate hypothermia group (green) comes from the pilot study run in 2014 and four animals were trained and tested in July 2016. The normothermia group (orange) consists of four rats from the pilot study, four from July 2016, two from August 2016, two from November 2016 and two from March 2017. The non-intervention group (gray) were combined with the normothermia group for the statistical analysis.

3.1 Failure to complete

Of the 23 animals trained between July 2016 and March 2017 (see figure 2), only 19 animals produced usable records at the retrieval Atlantis test. The failure to complete the experiment was in two cases due to death, one during normothermic anesthesia induction and another during the hypothermic procedure. Both were due to an overdose of isoflurane.

3.1.1 Loss of data points

Two additional animals from the pilot study failed to produce usable records at the retrieval Atlantis test due to tracking failure at the time of recording. Thus, the data points from four animals in total were lost due to tracking failure. Of these four animals, one came from the deep hypothermia group, one from the intermediate hypothermia group and the last two came from the normothermia group. Looking at the whole experimental period, from a total of 224 possible recorded, there was a loss of 31 timepoints (14%). These lost datapoints were lost completely at random due to recording failures.

3.2 Training period

3.2.1 Mean speed

To assess if there was a difference in physique between the groups during the training period and to make sure that the hour of hypothermia had not adversely affected the neuromuscular function, we compared average swim speed between the groups during training and before and after the intervention. The averaged speed (cm/sec) of the group in the Atlantis test at the beginning of each day is displayed in figure 3. There was no difference in average speed between the groups over the training period as assessed by a two-way mixed ANOVA. The data set did not meet the assumption of homogeneity of the covariance matrix, the analysis was carried out regardless. There was no interaction between the groups and training day, $F(5.13, 46.18) = 2.01$, $p = 0.94$. There was significant difference between the training days as assessed by their main effect, $F(2.56,46.18)=3.81$, $p < 0.05$. A Bonferroni post hoc test over the training days revealed that there only was a significant difference going from day two to day three. There was no significant difference between the groups during the training period $F(2, 18) = 1.59$, $p = 0.23$. Also, there was no significant difference in the speed between the groups at the baseline or retrieval trial as assessed by a one-way ANOVA, $F(2, 26) = 1.16$, $p = 0.94$ and $F(2, 25) = 0.31$, $p = 0.79$ respectively.

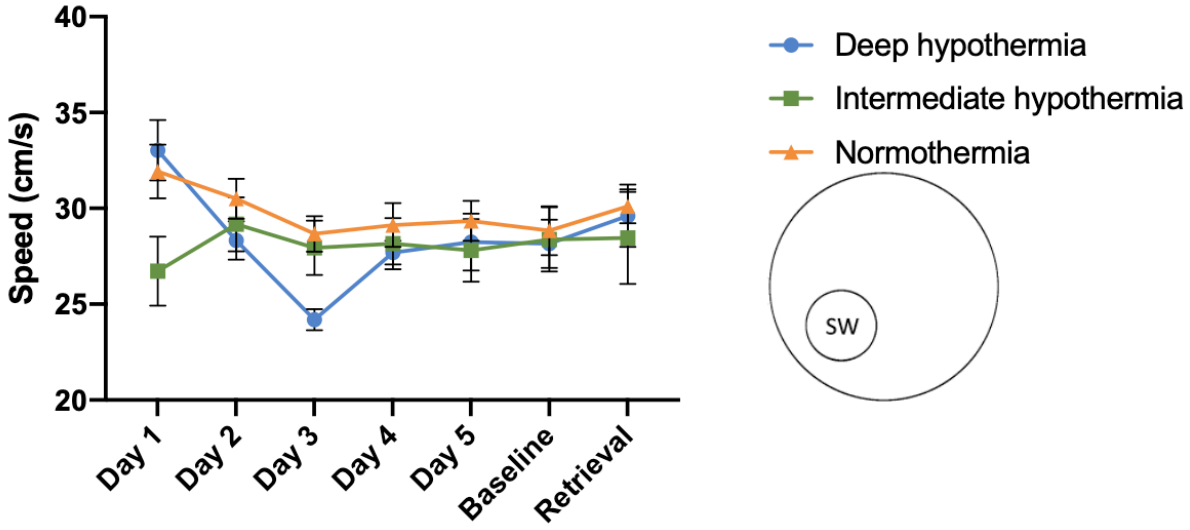


Figure 3 Average speed. Displays the average speed (mean ± SEM) per group for each Atlantis test of the training period and for the retrieval task. To assess if there was a difference in mean speed between the groups, a two-way mixed ANOVA was conducted. There was no significant interaction between the groups and training day, $F(5.13, 46.18) = 2.01$, $p = 0.94$. There was significant difference between the training days as assessed by the main effect $F(2.56,46.18)=3.81$, $p < 0.05$. A Bonferroni post hoc revealed that there only was a significant difference going from day two to day three. There was no significant difference between the groups during the training period $F(2, 18) = 1.59$, $p = 0.23$. There was no significant difference in the speed between the groups at the baseline or retrieval Atlantis test as assessed by a one-way ANOVA, $F(2, 26) = 1.16$, $p = 0.94$ and $F(2, 25) = 0.30$, $p = 0.79$ respectively.

3.2.2 Training results

During the training period of five days, the animals did learn the water maze task. This was assessed by recording the latencies to find the SW platform for all trials. These data were then averaged for each training day and group (figure 4). We used a two-way mixed ANOVA to assess if there was any difference in latency over the training period and if there was a difference between the groups. In the end there was no significant interaction between the groups and training days, $F(8, 120) = 1.10$, $P = 0.36$. There was significant difference between the different training days as assessed by its main effect $F(2.76,82.79)=44.83$, $p < 0.01$. A Bonferroni post hoc test over the training days was used to see where the difference lay. There was significant difference in latency between all days except going from day two to three and day four to five, ($p < 0.05$). The main effect of group was not significant, ($p = 0.40$).

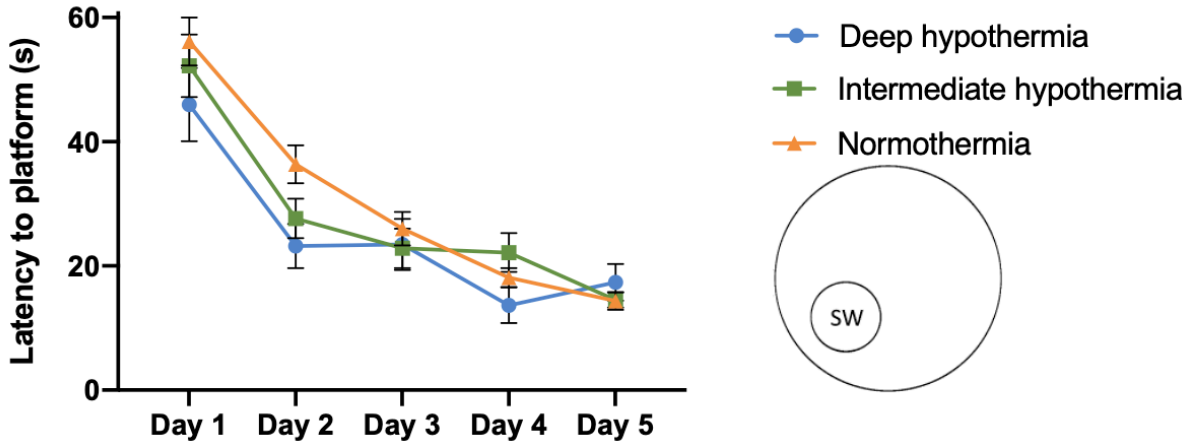


Figure 4 Latency to find platform. Displays the average latency (mean ± SEM) to find the SW platform location for each group during the training period of 5 days. There was no significant interaction between the groups and training days, F(8, 120) = 1.10, P = 0.36. There was a significant difference between the training days as assessed by its main effect F(2.76,82.79)=44.83, p < 0.01. A Bonferroni post hoc test showed a significant difference in latency between all days except going from day two to three and day four to five, (p < 0.05). The main effect of group was not significant, (p = 0.40).

3.3 Baseline Atlantis test

At baseline there was no significant difference between the groups comparing time spent in the target platform zone, SW, or in any of the other possible platform zones; NE, NW and SE. Figure 5 displays time spent in the platform zone (mean ± SEM) for all possible platform locations. The possible platform locations (NE, NW, SE, SW) are illustrated in the legend, with the target zone in solid line. The heatmaps display the locational occupancy summed across the group in the form of a 20 by 20 binned mesh. To assess if any of the groups were significantly different at baseline, a two-way ANOVA was conducted. The data set for the baseline Atlantis test did not meet the assumption of homogeneity of variance as the result of Levene's test was significant ($P < 0.01$), suggesting that there is inhomogeneity of variance in the data set. There was no interaction between the group affiliation and platform location, $F(6,104)=0.38$, $P = 0.89$). The main effect of platform location showed a significant difference between the platform locations, $F(3,104)=26.91$, $p < 0.01$, this was further analyzed for simple main effects which showed that the SW platform location was preferred across the groups, ($p < 0.05$). The main effect of group showed that there was no significant difference between the groups at this baseline trial when disregarding platform location, $F(2,104)=0.71$, $p = 0.49$.

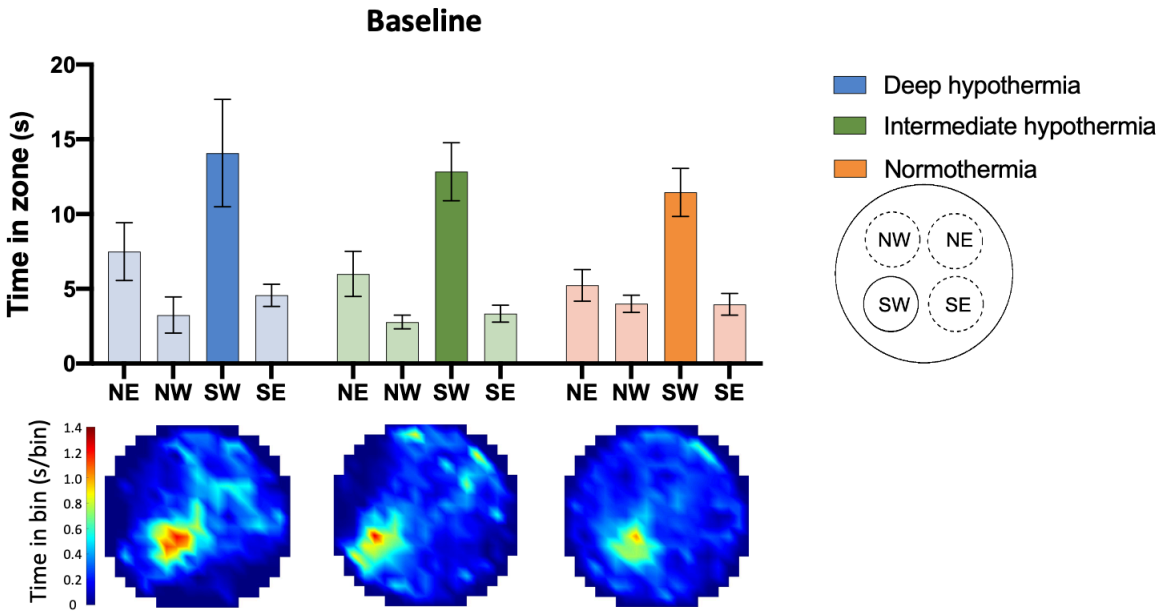


Figure 5 Baseline Atlantis test. Time spent in the platform zone (mean ± SEM) for all possible platform locations, the target location was in the SW. The Platform locations NE, NW, SE, SW are illustrated in the legend. The heatmaps display the locational occupancy summed across the group in the form of a 20 by 20 binned mesh. There was no significant difference between the groups at baseline, $F(2,104)=0.71$, $p < 0.49$. The main effect of platform location showed a significant difference between the platform locations, $F(3,104)=26.91$, $p < 0.01$, the pairwise comparison showed that the SW platform location was preferred across the groups, ($p < 0.05$). There was no interaction between the groups and platform location, $F(6,104)=0.38$, $P = 0.89$).

3.4 Retrieval Atlantis test

All the animals forgot the platform location. Figure 6 consists of the time in zone measurement for all platform positions in the retrieval Atlantis test, the target platform position was SW. To assess if there was a difference in the animals' memory of the task at retrieval a two-way ANOVA was conducted. The main effects of group showed that there was no significant difference between the groups when not accounting for platform location, $F(2,100)=25.97$, $p = 0.19$. The main effects of platform location on the other hand showed significant difference, $F(3,100)=98.76$, $p < 0.05$, further simple main effects showed only the NE vs NW platform location for the deep hypothermia group as significant, $p < 0.05$. There was no interaction between the group affiliation and platform location, $F(6,100)=11.76$, $p < 0.60$.

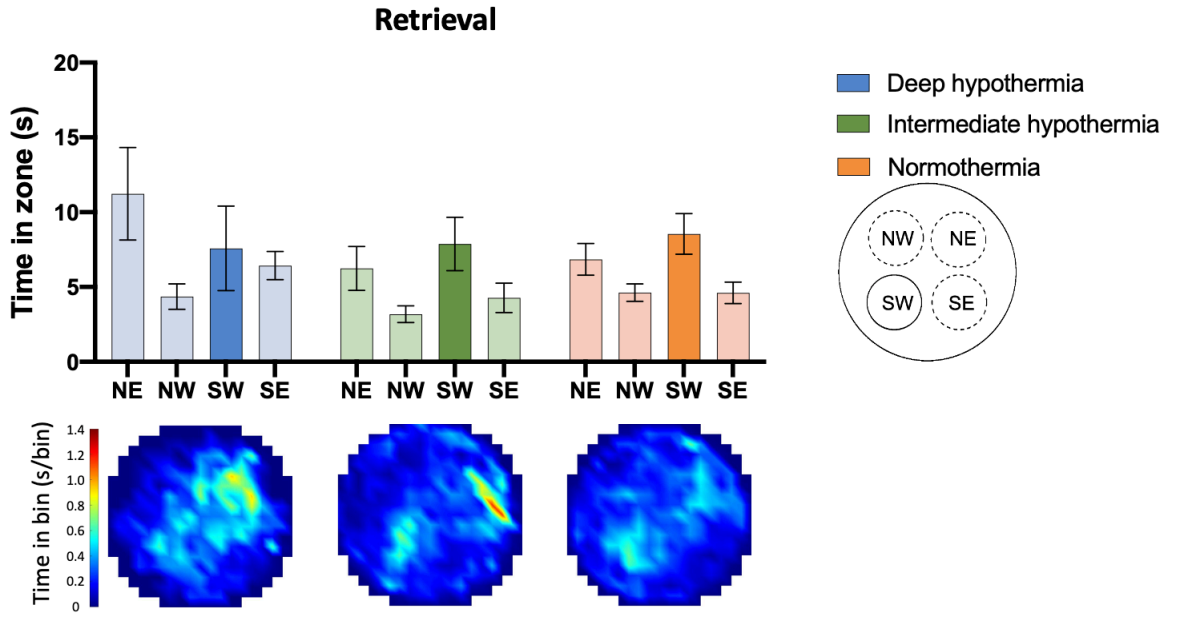
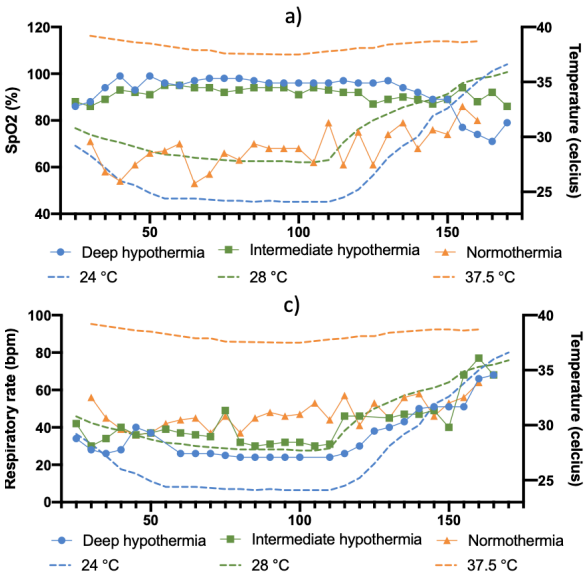


Figure 6 Retrieval. Time in zone measurement (mean ± SEM) for all possible platform locations at the retrieval Atlantis Test, the target platform position was SW. There was no significant difference between the groups, $F(2,100)=25.97$, $p = 0.19$. There was a significant difference between the platform position, $F(3,100)=98.76$, $p < 0.05$, the simple main effects revealed only NE vs NW for the deep hypothermia group as significant. There was no interaction between the group affiliation and platform location, $F(6,100)=11.76$, $p < 0.60$.

3.5 Vital parameters

During the hypothermia and normothermia procedure, the vital data parameters were recorded every fifth minute. Figure 7, displays vital data recorded from a single animal of each group as an example of the physiological effect of hypothermia. The table (d), displays the mean values for each group during the cooling phase, the 60 minutes at target temperature and the rewarming phase. The same animal is presented across the graphs. All the graphs display core temperature on the right-hand side Y-axis. Graph a) displays the heart rate on the left-hand axis, graph b) displays the respiratory rate on the left-hand axis and graph c) displays the SpO2 data on the left-hand axis. The x-axis shows the time elapsed from the beginning of the induction and is starting at 25 minutes due to the practical nature of the induction and stabilizing process. The legends are presented below the graph, with the groups above marked with circle, square or triangle, and the target temperature for the animal below marked with a stippled line. These animals were representative for their whole group. Table d) contains the mean values for each group during cooling, at target temperature and during rewarming.



d)

	Part of procedure	Deep hypothermia	Intermediate hypothermia	Normothermia
Temperature	Target	24.30	28.08	36.95
SpO2	Cooling	82.72	85.79	78.59
	Target	90.69	86.08	76.80
	Warming	85.59	83.64	75.06
Heart rate	Cooling	347.12	326.15	419.27
	Target	191.76	268.24	406.65
	Warming	342.22	323.54	376.28
Respiratory rate	Cooling	45.30	46.90	58.74
	Target	25.96	39.19	60.30
	Warming	61.63	51.45	59.14

Figure 7 Vital parameters. The graphs display vital data recorded every fifth minute from a single animal of each group as an example of the physiological effect of hypothermia. The Table displays the mean values for each group during the cooling phase, the 60 minutes at target temperature and the rewarming phase. The same animal is presented across the graphs. All the graphs display core temperature on the right-hand y-axis. Graph a) displays the SpO2, graph b) displays the heart rate and graph c) displays the respiratory rate on the left hand y-axis. The x-axis shows the time elapsed from the beginning of the induction. The legends are presented below the graph, with the groups above marked with circle, square or triangle, and the target temperature for the animal below marked with a stippled line.

3.6 Learners and non-learners

To further investigate the effect of hypothermia on long-term memory, we divided the animals into learners and non-learners based on their memory performance. Learners were defined as reaching a criterion of spending more than 10 seconds in the target zone during the baseline Atlantis test. A total of 18 animals were identified as learners, three deep hypothermia animals, seven intermediate hypothermia animals and eight normothermia animals. Due to the low number of deep hypothermia animals, we combined all the hypothermic animals into one group and compared it to the normothermic animals using a two-way ANOVA to see if the selection had made a difference to the performance in the task at the baseline and retrieval Atlantis test.

The baseline Atlantis test for the learners is presented in figure 8, which displays time in zone measurements (mean ± SEM) for all platform location. To assess if there was a significant

difference between the groups, a Two-way ANOVA was used. The two-way ANOVA revealed no interaction between the group affiliation and platform location, $F(3,64)=6.27$, $p = 0.63$. Furthermore, the main effect of group showed no significant difference between the groups $F(1,64)=0.89$, $p = 0.77$). But the main effect of platform location was significant, $F(3,64)=674.71$, $p < 0.05$. The simple main effect showed the SW platform location to be the prominent one, $p < 0.05$.

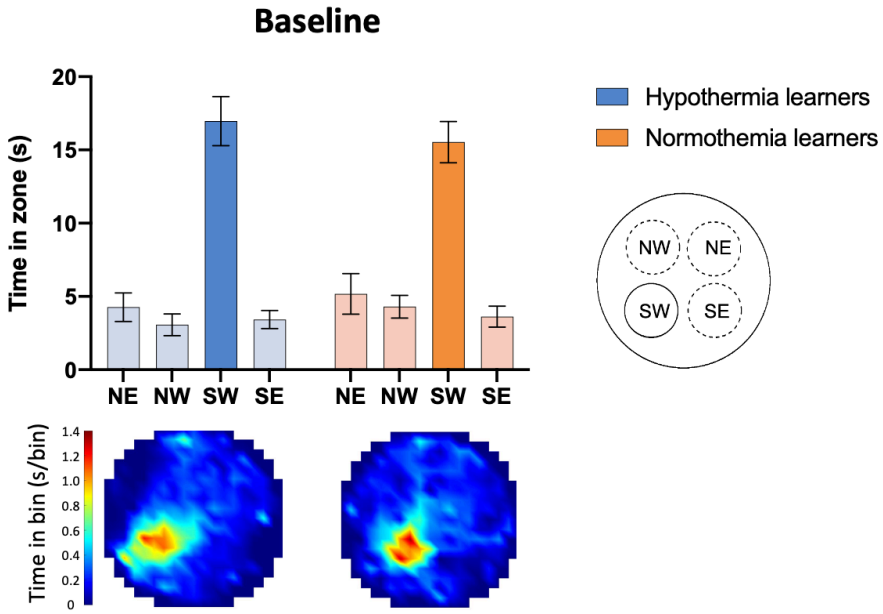


Figure 8 Baseline Atlantis test in rats with the highest memory performance (learners). Time in zone measurements (mean ± SEM) at the baseline trial for all platform locations after filtering out all animals with less than 10 sec in the SW zone at baseline is displayed. There was no interaction between the groups and platform location, $F(3,64)=6.27$, $p = 0.63$. The main effect of group showed no significant difference $F(1,64)=0.89$, $p = 0.77$). But the main effect of platform location was significant, $F(3,64)=674.71$, $p < 0.05$. The simple main effects revealed the SW platform location, $p < 0.05$.

The retrieval Atlantis test for the learners is presented in figure 9. As with the baseline Atlantis test, a two-way ANOVA was conducted to assess if there was any difference between the groups and platform location. There was no significant difference when looking at the main effect of group affiliation, $F(3,52)=0.03$, $p < 0.974$, platform location, $F(3,52)=62.11$, $p < 0.07$, or when comparing group affiliation in relation to platform location, $F(3,52)=3.47$, $p < 0.936$.

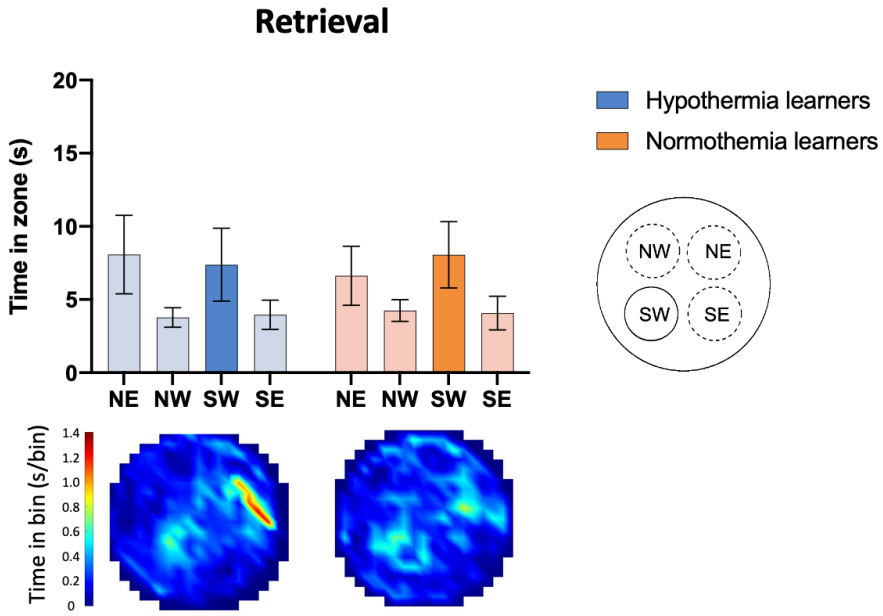


Figure 9 Retrieval Atlantis test of learners. There was no significant difference when looking at group affiliation, $F(3,52)=0.03$, $p < 0.974$, platform location, $F(3,52)=62.11$, $p < 0.07$, or significant interaction of group affiliation and platform location, $F(3,52)=3.47$, $p < 0.936$.

3.7 Distance to platform

Figure 10 displays the mean distance to the SW platform for each Atlantis trial. This is presented as an example of the other analysis method we used for the water maze, namely mean cumulated distance to platform. We used a custom MATLAB scrip to calculate the distance between the animal and the platform center for all recorded animal positions. No statistical tests were run on these results.

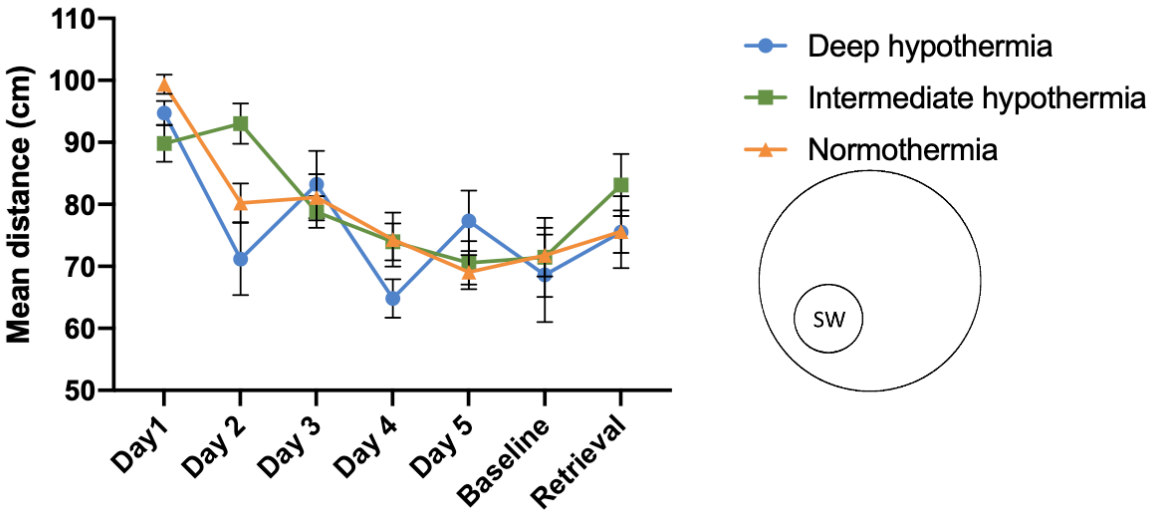


Figure 10 Distance to platform. Displays the mean distance (mean ± SEM) to SW platform for the Atlantis trial at each day. This was another way we tried to analyze the data.

3.8 Histology

A total of 22 brains were sliced. As we wanted to view as much of the brain as possible, we sliced through the whole brain as described above. Each brain resulted in approximately eight super frost plus slides with four slices per slide. Thus, an approximate total of 704 slices or 96 slides were stained with FJC, with some brains also stained with cresyl violet stain. Two duplicates of each slice were stored in separate Eppendorf tubes on a 10% DMSO solution and then frozen to serve as backup.

During analysis, it became clear that that there were no positive FJC cells in in any slices, only background noise. Figure 11 shows representative examples of the histology (hippocampus) from each experimental group. The right column contains FJC stained slices, while the right column contains cresyl violet stained slices. There are no fluorescent cells in the FJC slices, only background noise and artefacts from the staining procedure. The cresyl violet stains show normal morphology and so corroborates the finding from the FJC staining. The Positive control brains, lesioned with ibotenic acid, were destroyed by freezing artefacts during transport and were thus not usable.

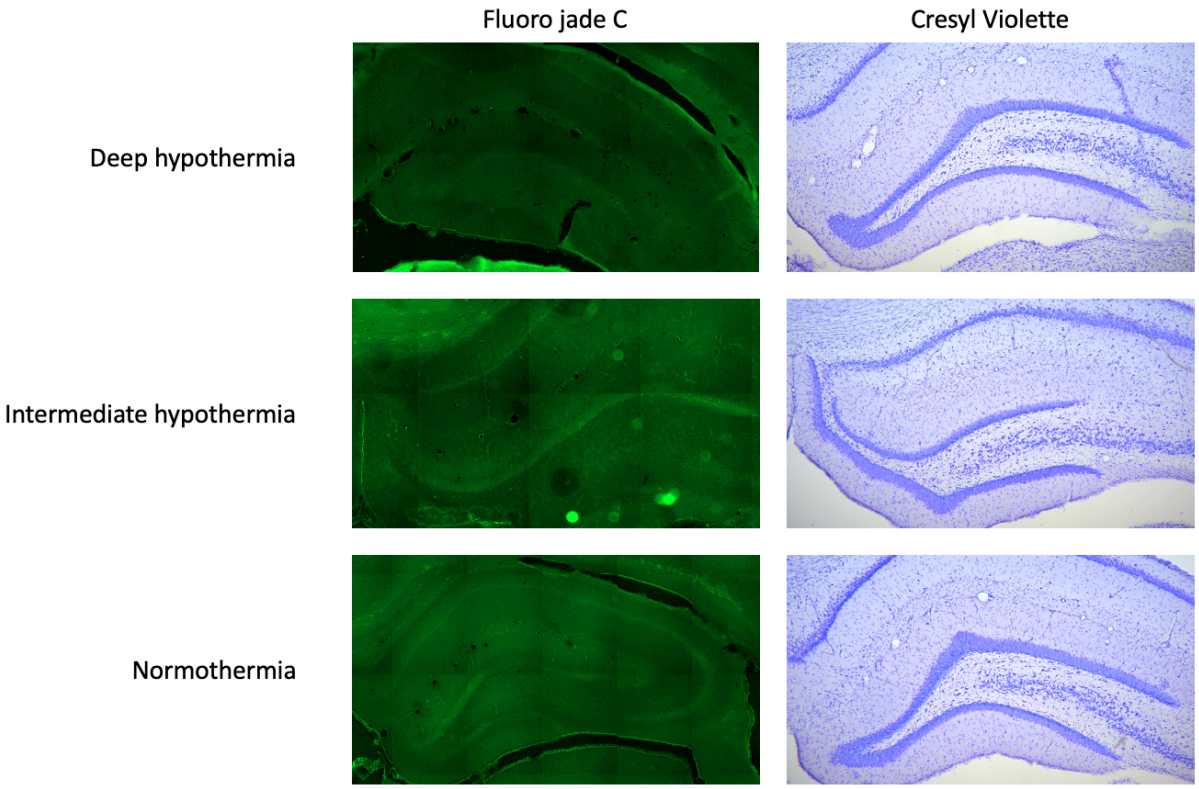


Figure 11 Histology. The figure contains a picture of the right hippocampus from an animal of each group. The right column displays an FJC stained hippocampus, and the right displays a cresyl violet stained hippocampus. There are no FJC positive cells in the FJC pictures and the morphology seen in the cresyl violet pictures is intact.

4 Discussion

We have in this study examined if one hour of hypothermia at either 24, 26, 28, 30 or 32 °C can induce retrograde amnesia for the water mase task, compared to normothermia. We found no difference between the normothermic or hypothermic groups in performance. We did not find signs of histological damage using the Fluoro jade C stain, but were unable to draw a conclusion due to a lack of verification of the protocol.

The project was severely hampered by the ongoing construction work in the vicinity of the animal facility. And in 2017, the lab had to close and is still not open at the time of writing, hence most of the planned experiments were not performed. The relatively low number of animals in each group might increase the chance for a type two error because the effect we are studying might be smaller than our power allows us to detect.

4.1 Behavioral results

The animals showed a decreasing latency to find the platform during the training period, as can be seen in figure 2. The two-way mixed ANOVA showed no significant difference between the groups in relation to performance over the training days. This suggests that the animals learned the water maze task and that there was no difference between the groups. Over the course of training, we registered individual variability in performance, but also within subjects, there were fluctuations form day to day and from the first to the last trial in how precise the animals searched. This was to be expected as the first trial of the day is dependent on the long-term memory consolidation of the platform location from the day before. The last trial of the day on the other hand is only dependent on the animal remembering the platform location from the last trial, 30 seconds before, and thus is mostly dependent on the short term memory (21). But as there was no difference between the groups at the baseline trial, we can conclude that the animals learned the task comparably well.

The question we wanted to answer in the study was if hypothermia could affect long term spatial memory through retrograde amnesia. Based on the results from the retrieval trial, we can conclude that there was no difference in how much the hypothermic groups remembered compared to the normothermic group. We had only a very low number of animals in the non-intervention group, which did not go through either anesthesia or hypothermia, therefor we cannot draw the conclusion that hypothermia or anesthesia in itself induces more retrograde amnesia than the natural decay of memory. Thus, we can only conclude that our results suggest that hypothermia induces no more retrograde amnesia than anesthesia. Hypothermia has earlier been used to induce retrograde amnesia for a single trial passive aversion task (22-24). The postulated mechanism for this is that hypothermia disrupts the consolidation process and thus induces retrograde amnesia. Earlier studies suggest difficulty in inducing retrograde amnesia for an overtrained task which might explain why we did not see any difference between the hypothermic or normothermic animals (34, 35). An alternative explanation could be that all groups experienced an equal amount of retrograde amnesia from the anesthesia. This is highly unlikely due to the anesthesia and the water maze task being a well-documented combination. Thus, the most likely explanation for the lack of group difference we see in the retrieval trial is that the hypothermia did not induce retrograde amnesia. This might be either because the task was overtrained, because the stimulus was too weak, because

the amnestic effect of hypothermia requires the animal to be conscious, like in the experiments conducted by Riccio et al.(22-24, 36-38), or just because the effect is too small for our design or power to examine.

4.2 Construction noise

We observed an unexpected bias in the behavioral data with wide standard errors and a search preference towards the NE platform position in the baseline and retrieval Atlantis tests. We can see in the baseline (figure 5) and retrieval Atlantis test (figure 6) that the deep hypothermia group had the greatest bias towards NE among the groups. In the retrieval trial, they even showed higher affinity for NE than for the target platform position, SW.

A possible explanation for the biased search pattern could be due to elements of the experimental setup and design. The door to the next room, where the drying cage and technician were located, could not be closed due to cable runs, and hence provided an attractive odorant cue in NE (figure 1). Also, the animals might have become overtrained in the Atlantis task during the training period and therefor gave up the search too early in the trial, switching to alternative escape strategies. But both of these sources of bias should be detectable in all groups and in similar experiments performed in the same setup, which was not the case. On the contrary, a pilot batch of rats trained with the exact same protocol and setup in 2014 and in another experiment (39), did not show any search bias towards NE. Thus, we do not think the bias could result from the experiment design.

Our main suspected source of the bias is that it resulted from the renovation of the neighboring building (se figure 1 for layout of lab in relation to construction work). The construction noise consisted of hammering, drilling and explosions both during experiments and during rest. The noise and vibrations came at random timepoints and there was mostly no warning. The animal facility, being pure concrete, transmitted the noise and vibrations to such a degree that we observed rippling during some of the water maze trials. This could explain why we do not see the same bias in the other groups, as the noise would increase as the construction intensified and came closer to the animal facility in the year before the animal

facility closed down. As figure 2 illustrates, the deep hypothermia group was all run in the beginning of 2017, just before the animal lab closed down, a period dominated by construction noise

There is strong evidence that laboratory animals have an adverse reaction to construction noise and vibration, more so than the technicians working with the animals. In 2011 Raff et al found that animals housed in an animal facility while construction work was being conducted 30 meters away from the facility showed double or even triple the levels of plasma ACTH, corticosterone and aldosterone compared to pre and post construction levels (40). These are important substances of the response system to stress in most animals, known as the hypothalamus, pituitary and adrenal axis, HPA-axis (40-43). An elevation of these substances is widely accepted to be related to heightened alertness, bodily feeling of stress and heightened blood pressure. Also, the hippocampus has a great amount of corticosterone receptors and a lot of research points to the fact that it is affected by increased levels over time (41, 44, 45). Meaning at the very least that we could expect it to have affected the experiments.

4.2.1 Filtering for learners

We theorized that due to the construction noise, there might be animals in the groups that had not learned the task. To see how this might have affected the results, we filtered the animals based on the time spent in target zone at the baseline trial by removing all animals who had spent less than 10 sec within the target zone. This way we would only be comparing the animals we knew had learned the task. The filtering was done using a custom MATLAB script. Due to the filtering we had to combine the hypothermic groups as there would have been too few in the deep hypothermia group to run any meaningful statistics. In the end there was no difference between the normothermia or hypothermia group after filtration at either the baseline or retrieval Atlantis test. This suggests that the animals that did not perform as well in the baseline trial, did not affect the end result. We also tried to filter for time of inclusion to see if there was any difference between the animals included in 2016 versus 2017, due to the probable increase in construction noise. The result is not presented in this paper, but it did not show any difference between inclusion time.

4.3 Vital parameters

During the hypothermia procedure vital parameters were recorded every fifth minute. As can be seen in the graphs in figure 7 the hypothermia induces some distinct physiological effects on heart rate, oxygen saturation (SpO2) and respiratory rate. As soon as the hypothermia was initiated, the heart rate and the respiratory rate drops, the spO2 rises. The heartrate of the normothermic animals can be seen to be around 370 bpm, compared to the 190 bpm of the hypothermic group during the target temperature period. This lowering of heartrate is a known phenomenon and is believed to emerge from the hypothermic effect on the pacemaker cells leading to sinusoidal bradycardia through a combination of a lowering of the sympathetic and increase of the parasympathetic signaling (46, 47). This is because the pacemaker cells intrinsic rate is 100 bpm.

The spO2 of the normothermic animals was stable close to 70%, while the hypothermic group pivoted around 90% and actually increased as the hypothermia was induced. This increase in spO2 could be explained by a combination of the lower oxygen demand, due to the decreasing energy consumption of the brain by approximately 5-10% per °C <37.5 (15), and the left shift of the oxygen dissociation curve. Pulse oximetry is an indirect measuring method and we are measuring the saturation of oxidized iron in the hemoglobin. Thus the left shift in the dissociation curve will give a higher saturation reading for a lower partial pressure of oxygen in the blood. Other studies in dogs have found the oxygen demand and delivery to be equally reduced during hypothermia, suggesting that despite the low respiratory rate the hypothermic group was most likely not hypoxic during the procedure (48).

The lowering of the respiratory rate has been found to be dependent on the temperature of the medulla oblongata. Suggesting that our method is indeed cooling the brain as well as the rest of the body (49).

4.4 Histology

Our plan was that FJC would indicate if the hypothermia was damaging or destroying braincells in the hippocampus. This would allow us to do two things. First, it would allow us

to connect the possible behavioral effects to a cellular effect and thus strengthen our hypothesis. And second, we would have strong evidence that hypothermia had a damaging effect on the brain. Since we did not find any change in the behavioral test, we only wanted to use the FJC histology to check if hypothermia induces damage to the neurons.

4.4.1 Missing positive control

In the histological pilot project (50), not to be confused with the behavioral pilot, we tried to reproduce the FJC procedure from Schmued et al 2005 (32). The results had to be concluded as inconclusive due to both the lack of FJC positive cells and positive controls. The lack of positive controls was again due to the closing of the animal facility. After the histological pilot we managed to procure rats injected with ibotenic acid in the hippocampus. This would allow us to verify if the protocol was working and thus allow us to conclude if the lack of FJC positive cells in the hypothermic animals was an actual finding or resulting from a fault of the protocol. Unfortunately, the brains were frozen during transport from Oslo to Tromsø, and were destroyed by freezing artifacts. Thus, we cannot draw any conclusion based on the FJC histology, since we cannot conclude that what we are seeing is not an artefact of the protocol. This is unfortunate since staining of the rest of the hypothermia exposed brains revealed no FJC positive cells either. This could suggest that there might not be any damage, which would complement the behavioral findings of this experiment.

The histological records of one of the rats is missing due to air bubbles in the perfusion line. The air bobble was pumped into the arterial system and created a thrombus and prevent continued perfusion. Thus the brain was unperfused and full of blood cells and plasma, which would be a source of major histological artefacts as most stains also bind to the blood cells and the proteins in the plasma as well as their target. The other problem is that an unperfused brain has the consistence of a slightly dense jelly and is easily ruined during extraction. The unperfused brain in question was discarded due to it being ruined during extraction.

4.5 Methods discussion

4.5.1 Anesthesia

The combination of anesthesia and hypothermia presented a difficult part of the project. After stable anesthesia had been reached at the beginning of the experiment, the flow and concentration of the isoflurane had to be titrated down to a lower level. As almost all excretion of isoflurane happens through respiration with only fractions being broken down in the liver (51). The low respiratory rate in combination with the very slow heart rate meant that there was an accumulation of isoflurane in the fatty tissues of the body, but specifically the fatty tissue of the brain. Thus, as the animals reached target temperature, we had to turn down the concentration of isoflurane almost to the lowest values the machine would allow in order to avoid lethal doses. This left only the flow value to adjust the amount of isoflurane administered leaving little room to make fine adjustments. This was the reason for the death in the hypothermia group. As the rat cooled, the gas concentration was kept too high and the respiration eventually stopped.

Size was another factor that affected the anesthesia of all groups. The smaller animals needed less induction and less maintenance anesthesia than their bigger counterparts. They were easier to keep under stable anesthesia; they would not fluctuate in sedation and they seemed to recover quicker. This was most likely due to the accumulation of the isoflurane in the fatty tissue of the animals, there would be some internal slow release of anesthetics that probably meant that any adjustment in gas saturation took longer to gain effect in the animal. This made the anesthesia more difficult.

4.5.2 The ibotenic acid injected animals

During injection of the ibotenic acid, there was no apparent sign of the drug affecting the animals. But when the animals were taken off the isoflurane anesthetics, they spent almost an hour waking up, normally this only takes two to three minutes. This is a known side effect of the ibotenic acid, although rarely described in the literature. Eventually the animals woke up, and during follow up they showed no sign of being affected by the drug.

4.6 Future directions

Many details are still unrevealed in understanding the effect of hypothermia on the brain. We had originally planned to run one male and one female series consisting of four groups of 8 rats cooled to either 30, 28, 26 or 24 °C. This way we would have a large population of hypothermic animals differentiated over temperature and sex. We would also be able to test if there were any accumulated effects of lower temperatures, a sort of dose response. In future research the inclusion of female subjects will be of vital importance. Especially since research has shown that female and male rats react differently to hypothermia, specifically when it comes to spatial memory. In 2014 Elmarzouki found that repeated cold exposures increased the females performance in the water maze while their male counterparts decreased performance with the same exposure (52). Anecdotally there is research suggesting a difference in response to hypothermia between the sexes also in humans. As in the Præstø accident of 2011 in Denmark. On a paddling trip in the middle of February, 13 teenaged students fell into the 2°C cold water, all wearing life vests. During their swim to shore, seven experienced cardiac arrest due to hypothermia, while six did not. Of the ones not experiencing cardiac arrest there were five females and one male. Even though Wanschker et al concluded that the difference lay in the body mass index, as the females not experiencing cardiac arrest had a significantly higher index than the males. It is still an interesting example of the importance of diversifying the studied populations. In the end all 13 students survived (53).

Although it is not mentioned in this paper, we did try to sample blood from the tail vain and through jugular vein catheterization during the hypothermia for blood gas measurements. As we did not ventilate the animals, this could tell us how the blood oxygen levels responded to the hypothermia. We would also be able to monitor the electrolytes and the pH of the blood. We were unsuccessful in sampling the tail vein due to the peripheral vasoconstriction inferred by the hypothermia, and the jugular vein catheterization due to it being a difficult procedure and due to time constraints for the total procedure as cooling would have to be commenced after the surgery. But this would only tell us about the blood oxygen and electrolyte levels, when what we are most interested in is what is happening in the tissue of the brain. In future studies examining this by the use of a micro dialysis implanted into the hippocampus during the hypothermia procedure could yield interesting results. This would have to be done in an

acute experiment but might tell us a lot about the what is going on in the brain tissue during hypothermia.

It would also be interesting to recreate Riccio's findings with the single trial passive avoidance task but with our model for hypothermia during anesthesia. As Vardaris, Gaebelein and Riccio postulated in 1973 that the retrograde amnesia resulting from hypothermia was induced through seizure activity (38). It might be that our use of an anesthetic agent stops such a seizure activity and thus attenuates the retrograde amnesia (54). Another possible explanation for their result is the choice strain, as the Long Evans rats used in our experiment is more robust than the albino Sprague–Dawley rats employed by Vardaris (55). Future studies should investigate in detail how hypothermia affects the brain. It should also seek to describe the mechanisms behind the dichotomous findings of neuroprotection and adverse effects seen in laboratory and clinical studies.

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6 Appendix

6.1 Appendix 1 – Stress scoring

Rat number: Start date: End date:

Parameter	Baseline			Perioperative D6		Recovery				Forklaring:
	D1	D2	D3	preop	postop	D4 t = 24	D5	D6	D7	D8	
Respirasjonsfrekvens (antall/min)											Telles og noteres daglig, mellom 60-120 gir 1 poeng, utenfor gir 2
Temp (grader C)											Måles rectalt, krever anestesi. Inngår ikke i totalt poengberegning
Urinproduksjon (Ja/Ikke observert)											Postoperativt legges ratten på et papirhåndkle som senere sjekkes for spor av urin.
Vekt (g)											Veies daglig. 1: > = 100% av utgangsvekt 2: 91-99% av utgangsvekt 3: = eller < 91% av utgangsvekt
Matinntak (g/antall)											Telle gjenværende mengde før neste føring og ettergi til 20 pellets ved føring .1: 80-100% av preop inntak 2: 60-80% av preop inntak 3: <60% av preop inntak
Avføring antall (boll/poeng											Avføring telles: 1: normal ved f.unn av > 5 feces. 2: sannsynlig obstipasjon ved < 5 stk .3: Fravær av avføring. Svær obstipasjon mistenkes også ved løs, rennende avføring (diaré).
Pels kvalitativ/poeng											Pels bedømmes kvalitativt. 1: normal, stelt. 2: **stedvis** stritttende pels og flass (begrenset område) 3: **generelt** stritttende, flassende pels
Nevrologi NDS/poeng											Nevrologisk vurdering etter NDS: 1 NDS = 86, 2: NDS 75-86, 3: NDS <75 eller epileptiske anfall. Ved stabil høy NDS som ikke krever tiltak D8-10 kan man vurdere å avstå fra videre scoring
Sosial skår kvalitativ/poeng											Sosial adferd observeres 5 min: 1: normal - rotte viser adekvat respons (e.g vil unslippe) på menneskelig kontakt. 2: Ikke-ygl/gjm angående respons på håndtering, kan sees forbigående under oppvåkning etter anestesi. 3: piper, graver, sitter immobil i et hjørne, forsøker å bite
Total skår											Total skår > 10 skal dyret avlives straks, > 7 kontakt veterinær

Endepunkter perioperativt og under recovery
Den første timen etter revarmning observeres dyret kontinuerlig, deretter hver time i til sammen 6 timer.
Tegn til hjemmeskade som skal føre til at forsøket avbrytes umiddelbart: lysstive pupiller, ingen smertereaksjon, fravær av spontanventilasjon, epileptiske anfall.
Dersom slike tegn oppstår på et senere tidspunkt under recoveryperioden skal dyret avlives.

	<-- betyr viktig
	<-- betyr at faller bort

Poengscore
Observasjoner D1-D3 noteres og brukes som baseline for vurdering av dyrets tilstand under recovery D4-D8.
Lav score indikerer god helse, høyere score indikerer dårlig helse/mistrivsel. Minimum score er 5 poeng, maksimum score er 15 poeng.
Ved oppnådd enkeltskår >= 3, eller total skår > 10 skal dyret avlives straks
Ved oppnådd 2'er kontaktes veterinær for vurdering (dvs total skår >7)

Eksempel på bruk
En rotte som hyperventilerer og beveger seg mindre spontant vil få NDS = 81 og dermed 2 i poengberegningen. Denne skal altså vurderes sammen med veterinær.
En rotte som ikke eksplorerer, men sitter i hjørnet/piper/graver/biter under hele observasjonstiden har tegn til vedvarende stress. Den får 3 i poengberegningen - skal avlives.

6.2 Appendix 2 – Neurological deficit score

Neurodeficit scoring for rats (normal = 80; Good outcome > 60; brain dead = 0)

All scores are assessed in home cage except "balance on beam"

(A) General behavioral deficit Total score 19	Points	10		6		5	3		1	0	Assessment
Consciousness		Normal				Stuporous				Unresponsive	Attempt to explore spontaneously?
Arousal Eyes open							Spontaneously	To pain		no eye opening	Pain stimulus: limb pinch
Respiration				Normal			hypo/hypervent			absent	Normal RR: 60-120/min
(B) Brain-stem function Total score 21											
Olfaction: response to smell of food							Present			Absent	Sniffing crumbs of cereal put in home cage
Vision: head movement to light							Present			Absent	Following light from flashlight
pupillary light reflex							Present			Absent	Using a flashlight, look for pupillary constriction
Corneal reflex							Present			Absent	Using a flashlight, look for reflex blinking when exposed
Hearing							Present			Absent	Startle or turn to clapping of hands
Whisker stimulation reflex							Present			Absent	A blunt stick is moved toward the whiskers from the rear of the animal, avoid entering the visual fields.
Swallowing liquids or solids							Present			Absent	Observed in home cage
(C) Motor assessment Total score: 6											
Strength right side							Normal	slow/weak		no movement	Observe for 5 min in home cage and during motorfunction testing (E). Look for Symmetrical movment. Normal: limbs extend fully on one side
Strenght left side							Normal	slow/weak		no movement	
(D) Sensory assessment Total score:12											
Pain withdrawal right side				Brisk			Weak/abnormal			no withdrawal	Limb pinch. Abnormal response may be extension or flexion posture
Pain withdrawal left side				Brisk			Weak/abnormal			no withdrawal	
(E) Motor function Total score: 12											
Gait coordination							Normal	Ataxia		circular movment	Observe spontaneous movement, circular movment = Hemiplegia
Balance on beam				Normal			Reduced	Abnormal		Absent	Normal: > 50% of preprocedure balancing time Reduced: < 50% of preprocedure balancing time Abnormal: can balance for a short amount of time Absent: falls off immediately
Righting reflex							Normal	Abnormal		Absent	Animal placed on its back is able to correct to upright position
(F) Behavior Total score: 6											
Spontaneous exploration							Normal	Reduced		Immobile	Observe 5 min in home cage. Normal: explores and approaches <= 3 walls of the cage; Reduced: aproaches > 3 sides, hesitates to move, reaches >= 1 upper rim; Immobile: Rat does not rise up at all, barely moves and/or rat does not move.
Anxiety							None	both/neither		anxious	Observe for 5 min in cage. None: grooming is present. Anxious: if defensive burying/digging or screaming. If neither or both is observed: both/neither
(G) Seizures (convulsive/non-convulsive) Total score: 10		No seizure				Focal seizure				General seizure	Focal seizure: Not general. General seizure: loss of consiousness and all motorfunction

Total: 10x__+6x___+3x___+1x___= ____ assesment: normal = 86; Good outcome > 80; brain dead = 0

Modified version based on NDS found in Katz 1995, Garcia 1995 and Jungwirth 2006

09.01.2017

6.3 Appendix 3 – MATLAB scripts

```
function AnalyzeWM(inputfile,outputfile)
% Main I/O function for data analysis
% Reads session list at this format:
%
% Rat directory/ratnumber
% Rat directory/ratnumber2
% ---

% Created by
% Vegard Heimly Brun
% UiT - The Arctic University of Norway
%
% Reworked by
% Håvar Marsteen
% Copyright (C) 2017 - UiT
% All Rights Reserved

fid = fopen(inputfile,'r','ieee-be');


if (fid == -1)
   error('Error: Could not open file %s',inputfile);
end

clear global;
global Allvaluesneeded;

global TableOfValues;
global ThresholdT41SW;
Allvaluesneeded = cell(1,22); %Declares the storage for all values
 from run
TableOfValues = strings(1,0);
TableOfValues(1) = "Rat";
ThresholdT41SW = 0;

%write headers to outputfile
trial = ["T1_","T9_","T17_","T25_","T33_","T41_","T42_"];
headings =
 ["Speed","Pathlength","TimeNE","TimeNW","TimeSW","TimeSE",...
    "MDistNE","MDistNW","MDistSW","MDistSE","CDistNE","CDistNW",...

  "CDistSW","CDistSE","RzoneCDistNE","RzoneCDistNW","RzoneCDistSW",...
    "RzoneCDistSE","LatancySW"];
headingRange = [1,6];
while headingRange ~= 0
    for trialnum = 1:length(trial)
        for headingNum = headingRange(1):headingRange(2)
            TableOfValues(size(TableOfValues,1)+1,1) = append(...
                trial(trialnum),headings(headingNum));
        end
    end
    if headingRange(1) == 1
```

```
        headingRange = [7,10];

    elseif headingRange(1) == 7
        headingRange = [11,14];
    elseif headingRange(1) == 11
        headingRange = [15,18];
    elseif headingRange(1) == 15
        headingRange = [19,19];
    else
        headingRange = 0;
    end
    TableOfValues(size(TableOfValues,1)+1,1) = " ";
end


while ~feof(fid)
        while 1
        ThresholdT41SW = 0;
        textline = fgetl(fid);

        if ~strcmp(textline(1:3), 'Rat')
           break;
        end

        ratname = textline(end-3:end);
        ratfilelocation = textline(5:end);
          %Uses step a through b to sort for variable being calculated
        a = [1,6];
        collum = size(TableOfValues,2)+1;
        counter = 1;
        while a ~= 0
              TableOfValues(1,collum) = ratname;
              trialFile = ["_1.pos","_9.pos","_17.pos","_25.pos",...
                  "_33.pos","_41.pos","_42.pos"];
                  for trialNum = 1:length(trialFile)
                      posfile = sprintf('%s%s',ratfilelocation,...
                          trialFile(trialNum));
                      counter =
 calculate_and_write_values_to_outputfile...
                              (a,collum,counter);
                  end
               if a(1) == 1
                   a = [7,10];
               elseif a(1) == 7
                   a = [11,14];
               elseif a(1) == 11
                   a = [15,18];
               elseif a(1) == 15
                   a = [19,19];
               else
                   a = 0;
               end
               counter = counter + 1;
               TableOfValues(counter,collum) = " ";
```

```
        end
    end
end


    function [counter] =
calculate_and_write_values_to_outputfile(range,...
           collum,counter)
          if exist(posfile,'file')
              [posx,posy,post,dist,wmspeed,mean_pathlength,...

cumulated_pathlength,zonetime,cumulated_ratzone_area,...
                  Latancy] = getwmpos(posfile,range,ThresholdT41SW);
          else
              sprintf('File does not exist')
               [posx,posy,post,dist,wmspeed,mean_pathlength,...

cumulated_pathlength,zonetime,cumulated_ratzone_area,...
                   Latancy] = getemptyvalues;
          end
          values_to_write = [wmspeed,dist,zonetime(1),zonetime(2),...
              zonetime(3),zonetime(4),mean_pathlength(1),...

mean_pathlength(2),mean_pathlength(3),mean_pathlength(4),...
              cumulated_pathlength(1),cumulated_pathlength(2),...
              cumulated_pathlength(3),cumulated_pathlength(4),...
              cumulated_ratzone_area(1),cumulated_ratzone_area(2),...

cumulated_ratzone_area(3),cumulated_ratzone_area(4),Latancy];
          for i = range(1):range(2)
              value_in_string = num2str(values_to_write(i));
              counter = counter + 1;
              TableOfValues(counter,collum) = value_in_string;
          end

         function [posx,posy,post,dist,wmspeed,mpathlength,...
                 cpathlength,zonetime,cumulated_ratzone_area,...
                 Latancy] = getemptyvalues
            wmspeed = NaN;
            mpathlength = [NaN NaN NaN NaN];
            cpathlength = [NaN NaN NaN NaN];
            cumulated_ratzone_area = [NaN NaN NaN NaN];
            zonetime = [NaN NaN NaN NaN];
             dist = NaN;
             posx = NaN;
             posy = NaN;
             post = NaN;
             Latancy = NaN;
          end


    end

writematrix(TableOfValues,outputfile);
```

```
fclose(fid);
Print_heatmap_grouplevel()
clear global;
end
```

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Table of Contents

Deep Hypothermia

Rat WMdata2016+17/2024
Rat WMdata2016+17/2025
Rat WMdata2016+17/2026
Rat WMdata2016+17/2027
Rat WMdata2016+17/2028
Rat WMdata2016+17/2029

Intermediate Hypothermia

Rat WMdata2016+17/2003
Rat WMdata2016+17/2006
Rat WMdata2016+17/2010
Rat WMdata2016+17/2017
Rat WMdata2016+17/2002
Rat WMdata2016+17/2007
Rat WMdata2016+17/2009
Rat WMdata2016+17/2015
Rat WMdata2016+17/2011
Rat WMdata2016+17/2013

Normothermia

Rat WMdata2016+17/2001
Rat WMdata2016+17/2004
Rat WMdata2016+17/2005
Rat WMdata2016+17/2008
Rat WMdata2016+17/2012
Rat WMdata2016+17/2014
Rat WMdata2016+17/2016
Rat WMdata2016+17/2018
Rat WMdata2016+17/2019
Rat WMdata2016+17/2020
Rat WMdata2016+17/2022
Rat WMdata2016+17/2023
Rat WMdata2016+17/2030
Rat WMdata2016+17/2031
Rat WMdata2016+17/2021
Rat WMdata2016+17/2032
Rat WMdata2016+17/2033

```
function [posx,posy,post,pathlength,meanspeed,mean_pformdist,...
    cumulated_pformdist,zonetime,cumulated_ratzone_area,...
    latancy] = getwmpos(posfile,range,maxlen)

%YOU HAVE TO DEFINE THE PLATFORM RADIUS AND TIME INTERVAL
%TO ANALYZE MANUALLY
global ThresholdT41SW;
platformradius = 5; % defines the radius of the platform in cm
zoneRadius = 30; %defines the radius of the zone to be analyzed in cm
%Define interval to analyse 25 Hz sampling rate means 60 sec = 1500
starttime = 25;
stoptime = 1500;


% This script finds watermaze and platform positons automatically from
% wst-file
setupfile = sprintf('%s%s',posfile(1:end-3),'wst');
NotWanted = ["2001","2002"]; %hardcoded filter due to fault in WST
 files
IfPilot = contains(setupfile, NotWanted);
if IfPilot
    setupfile = "WMdata2016+17/2010_1.wst";
end
fid3  = fopen(setupfile,'r','ieee-be');
    if (fid3 == -1)
       error(sprintf('Error: Could not open file %s',setupfile));
    end

text = textscan(fid3,'%s');
text = text{1};

fid3  = fclose(fid3);

%Find watermaze
OBL = regexp(text,'OuterBoundaryLeft[\s\.=]+(\d+)','tokens');
OBL = [OBL{:}]; OBL = str2double([OBL{:}]).';
OBR = regexp(text,'OuterBoundaryRight[\s\.=]+(\d+)','tokens');
OBR = [OBR{:}]; OBR = str2double([OBR{:}]).';
OBT = regexp(text,'OuterBoundaryTop[\s\.=]+(\d+)','tokens');
OBT = [OBT{:}]; OBT = str2double([OBT{:}]).';
OBB = regexp(text,'OuterBoundaryBottom[\s\.=]+(\d+)','tokens');
OBB = [OBB{:}]; OBB = str2double([OBB{:}]).';

WMcentrex = round(OBL+(OBR-OBL)/2);
WMcentrey = round(OBT+(OBB-OBT)/2);
WMradius = (-(WMcentrey-OBB)+(WMcentrex-OBL)+(WMcentrey-OBT)-...
    (WMcentrex-OBR))/4;

%Find NE platform position
NE1 = regexp(text,'Platform_1_0[\s\.=]+(\d+)','tokens');
NE1 = [NE1{:}]; NE1 = str2double([NE1{:}]).';
NE2 = regexp(text,'Platform_1_1[\s\.=]+(\d+)','tokens');
```

```
NE2 = [NE2{:}]; NE2 = str2double([NE2{:}]).';
NE3 = regexp(text,'Platform_1_2[\s\.=]+(\d+)','tokens');
NE3 = [NE3{:}]; NE3 = str2double([NE3{:}]).';
NE4 = regexp(text,'Platform_1_3[\s\.=]+(\d+)','tokens');
NE4 = [NE4{:}]; NE4 = str2double([NE4{:}]).';

NEplfx = round(NE1+(NE2-NE1)/2);
NEplfy = round(NE3+(NE4-NE3)/2);

%Find NW platform position
NW1 = regexp(text,'Platform_2_0[\s\.=]+(\d+)','tokens');
NW1 = [NW1{:}]; NW1 = str2double([NW1{:}]).';
NW2 = regexp(text,'Platform_2_1[\s\.=]+(\d+)','tokens');
NW2 = [NW2{:}]; NW2 = str2double([NW2{:}]).';
NW3 = regexp(text,'Platform_2_2[\s\.=]+(\d+)','tokens');
NW3 = [NW3{:}]; NW3 = str2double([NW3{:}]).';
NW4 = regexp(text,'Platform_2_3[\s\.=]+(\d+)','tokens');
NW4 = [NW4{:}]; NW4 = str2double([NW4{:}]).';

NWplfx = round(NW1+(NW2-NW1)/2);
NWplfy = round(NW3+(NW4-NW3)/2);

%Find SW platform position
SW1 = regexp(text,'Platform_3_0[\s\.=]+(\d+)','tokens');
SW1 = [SW1{:}]; SW1 = str2double([SW1{:}]).';
SW2 = regexp(text,'Platform_3_1[\s\.=]+(\d+)','tokens');
SW2 = [SW2{:}]; SW2 = str2double([SW2{:}]).';
SW3 = regexp(text,'Platform_3_2[\s\.=]+(\d+)','tokens');
SW3 = [SW3{:}]; SW3 = str2double([SW3{:}]).';
SW4 = regexp(text,'Platform_3_3[\s\.=]+(\d+)','tokens');
SW4 = [SW4{:}]; SW4 = str2double([SW4{:}]).';

SWplfx = round(SW1+(SW2-SW1)/2);
SWplfy = round(SW3+(SW4-SW3)/2);

%Find SE platform position
SE1 = regexp(text,'Platform_4_0[\s\.=]+(\d+)','tokens');
SE1 = [SE1{:}]; SE1 = str2double([SE1{:}]).';
SE2 = regexp(text,'Platform_4_1[\s\.=]+(\d+)','tokens');
SE2 = [SE2{:}]; SE2 = str2double([SE2{:}]).';
SE3 = regexp(text,'Platform_4_2[\s\.=]+(\d+)','tokens');
SE3 = [SE3{:}]; SE3 = str2double([SE3{:}]).';
SE4 = regexp(text,'Platform_4_3[\s\.=]+(\d+)','tokens');
SE4 = [SE4{:}]; SE4 = str2double([SE4{:}]).';

SEplfx = round(SE1+(SE2-SE1)/2);
SEplfy = round(SE3+(SE4-SE3)/2);

%Define mean_pformdist and zonetime
mean_pformdist = zeros(4,1);
zonetime = zeros(4,1);

%Open posfile
fid4 = fopen(posfile,'r','ieee-be');
```

```
if (fid4 == -1)
   error(sprintf('Error: Could not open file %s',posfile));
end

%Read from the posfile, this is hardcoded
for t = 1:6
   string = fgetl(fid4);
end
sample_end = sscanf(string,'%*s %u');
trialToShort = 0;  %variable to catch error trials shorter than 60
 seconds
if sample_end < stoptime
    stoptime = sample_end;
    trialToShort = 1;
end

% moves 25 lines further down in the file
for t = 1:25
 string = fgetl(fid4);
end
fseek(fid4,10,0); % jumps 10 bits forward
temp = ones((stoptime-starttime),8);
post = ones((stoptime-starttime),1);
for i = 1:starttime-1
    temp(i,1) = fread(fid4,1,'uint16');
    temp(i,2) = fread(fid4,1,'uint16');
end
for ind = 1:stoptime-starttime
    temp(ind,1) = fread(fid4,1,'uint16');
    temp(ind,2) = fread(fid4,1,'uint16');
    ind2 = find(temp(ind,:) == 1023);
    temp(ind,ind2) = NaN;
end


fclose(fid4);
   posx = temp(:,1);
   posy = temp(:,2);

clear temp;

for ii = 1:stoptime-starttime
    post(ii) = ii/25;   % Here the Time position variable is defined
end

%Interpolate NaN in positions (untracked pos)
fixedposx = fixgaps(posx);
fixedposy = fixgaps(posy);

%Calculates zonescores for all four platform location
[mean_pformdist(1),cumulated_pformdist(1),zonetime(1)...
    ,cumulated_ratzone_area(1)] =
 zonescores(NEplfx,NEplfy,fixedposx,...
    fixedposy);
```

```
[mean_pformdist(2),cumulated_pformdist(2),zonetime(2),...
    cumulated_ratzone_area(2)] =
 zonescores(NWplfx,NWplfy,fixedposx,...
    fixedposy);
[mean_pformdist(3),cumulated_pformdist(3),zonetime(3),...
    cumulated_ratzone_area(3),latancy] = zonescores(SWplfx,SWplfy,...
    fixedposx,fixedposy);
[mean_pformdist(4),cumulated_pformdist(4),zonetime(4),...
    cumulated_ratzone_area(4)] =
 zonescores(SEplfx,SEplfy,fixedposx,...
    fixedposy);

window = 5;  %Skips coordinates To remove noice from the speed and
 distance
    % resulting form slight changes where on the rat it is tracking

if range(1) == 1  %stops it from printing a new figure on every
 itteration
    %of calculate_and_write_values_to_outputfile from WM_analyse

    %creates folder to write plots and charts to
    foldername = sprintf('%s%s','Figures_for_me20/',posfile(15:18));
    if ~exist(foldername,'dir')
        status = mkdir('Figures_for_me20/',posfile(15:18));
    end

%     Creates figure of platform position and plots path, change
%     figure(...,'off') to figure(...,'on') to view during run. Also
 saves
%     png version in folder, using exportgraphics()
    clf; %clear figure
    figure('name', posfile(1:end-3),'visible','OFF')
    plot(fixedposx(1:window:stoptime-starttime),...
        -fixedposy(1:window:stoptime-starttime))
    viscircles([SWplfx, -SWplfy], (platformradius*440/200));
    viscircles([SWplfx, -SWplfy], (zoneRadius*440/200));
    viscircles([NEplfx, -NEplfy], (platformradius*440/200));
    viscircles([NEplfx, -NEplfy],
 (zoneRadius*440/200),'LineStyle','--');
    viscircles([SEplfx, -SEplfy], (platformradius*440/200));
    viscircles([SEplfx, -SEplfy],
 (zoneRadius*440/200),'LineStyle','--');
    viscircles([NWplfx, -NWplfy], (platformradius*440/200));
    viscircles([NWplfx, -NWplfy],
 (zoneRadius*440/200),'LineStyle','--');

    viscircles([WMcentrex, -WMcentrey], (WMradius));
    set(gca,'xtick',[],'ytick',[]);
    pause(1);

    NameFigure = sprintf('%s%s%s%s
%s','Figures_for_me20/',posfile(15:18),'/',posfile(15:end-3),'png');
    exportgraphics(gcf,NameFigure);
    close;
```

```
    NameFigure = sprintf('%s%s%s
%s',posfile(15:end-4),'_Heatmap.','jpg');
    HeatmapOfPath = Create_heatmap(fixedposx, fixedposy, OBL, OBR,
 OBB, OBT, 0, 1.4,posfile(15:18));
    NameFigure = sprintf('%s%s%s%s%s%s
%s','Figures_for_me20/',posfile(15:18),'/',posfile(15:end-4),'_Heatmap.','jpg');
    exportgraphics(gcf,NameFigure);
    close;
end

%Calculates Pathlength and mean speed
pathlength = 0;
for i = 1:window:(length(posx)-window)
%     for i= 251:1500
%    sett inn while xy isnot platformposition
    distx = abs(fixedposx(i)-fixedposx(i+window))*200/(OBB-OBT);
    disty = abs(fixedposy(i)-fixedposy(i+window))*200/(OBR-OBL);
    distance = sqrt(distx.^2+disty.^2);
    if ~isnan(distance)
        pathlength = pathlength + distance;
    end
    currentspeed(i)= distance/(post(i+1)-post(i));
%     pause(1)
end
meanspeed = mean(currentspeed,'omitnan'); %the mean speed in cm per
 sec

function
 [mean_plf_dist,cumulated_plf_dist,zonetime,cumulated_ratzone_area,latancy]
 = zonescores(plfx,plfy,posix,posiy)
    %Calculates platform distance in x and y direction and convertes
 to...
    %cm, then calculates the length of the resulting hypothenus
    conversionX = 200/(OBB-OBT);
    conversionY = 200/(OBR-OBL);
    pformdistx = abs(posix-plfx)*conversionX;
    pformdisty = abs(posiy-plfy)*conversionY;
    pldist = sqrt(pformdistx.^2+pformdisty.^2);
    pldist_squared = pldist.^2;

    %calc mean platform distance for atlantis normally 0-60 sec
    if length(pldist) < stoptime-starttime
        stoptime = length(pldist);
        sprintf('Warning, length of test was shorter than expected');
    end
    mean_plf_dist = mean(pldist,'omitnan');%omitnan to fix leadning
 NaNs in position tracking
    cumulated_plf_dist = sum(pldist,'omitnan');
    cumulated_ratzone_area = sum(pldist_squared,'omitnan');

    %calculate time within platform zone radius as defined above
    index_withinzone = pldist <= zoneRadius;
    zonetime = sum(index_withinzone)/25; %in seconds
```

```
    %calcuate latancy for SW platform, and filter/add to global group
 variable
    if plfx == SWplfx && plfy == SWplfy
        latancy = 60;
%         if range(1) == 1 && contains(posfile,"_41.pos") && zonetime
 >= 10 %uncomment to filter for 10 sec in trial 41
%             ThresholdT41SW = 1;
%         end
        if range(1) == 1 %&& ThresholdT41SW % uncomment to filter for
 10 sec on trial 41.

 Fill_up_the_globals(posfile,fixedposx,fixedposy,OBL,OBR,OBT,OBB);
            posfile
        end
        for L = 1:length(pldist)
            if pldist(L) <= (platformradius)
                latancy = L/25; %Latancy to first contact with
 platform position
                break
            end
        end
    end

end

% If the trial is too shorte, returns NaN for all variables.
if trialToShort == 1
    meanspeed = NaN;
    pathlength = NaN;
    zonetime = [NaN NaN NaN NaN];
    mean_pformdist = [NaN NaN NaN NaN];
    cumulated_pformdist = [NaN NaN NaN NaN];
    cumulated_ratzone_area = [NaN NaN NaN NaN];
    posx = NaN;
    posy = NaN;
    post = NaN;
    return
end

end
```

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```
function Fill_up_the_globals(RatFilename,ArrayofCoordinatesx,...
    ArrayofCoordinatesy,OBL,OBR,OBB,OBT)
global Allvaluesneeded;

% Sorts the animals into their respective group
DeepHypothermia = ["2024","2025","2026","2027","2028","2029"];
IntermediateHypothermia = ["2002","2003","2006","2007","2009",...
    "2010","2011","2013","2015","2017"];
Normothermia = ["2001","2004","2005","2008","2012","2014","2016",...
    "2018","2019","2020","2021","2022","2023","2030",...
    "2031","2032","2033"];
Rat_number = convertCharsToStrings(RatFilename(15:18));
GroupVariable = "";
if contains(Rat_number,DeepHypothermia)
    GroupVariable = 1;
elseif contains(Rat_number,IntermediateHypothermia)
    GroupVariable = 8; %change back to 8 to have all three groups
else
    GroupVariable = 15;
end


if RatFilename(19:end-4) == "_1" || RatFilename(19:end-4) == "_9"
    trialNumber = RatFilename(end-4);
else
    trialNumber = RatFilename(end-5:end-4);
end

% Whole number division to sort based on trial number
a = str2double(trialNumber);
step = int16(8);
if a == 42
    a = idivide(a ,step) + 1;
else
    a = idivide(a ,step);
end

Collum = double(GroupVariable+a);

sizeOfArrayofCoordinates = length(ArrayofCoordinatesx);
sizeOfOveralltablex = length(Allvaluesneeded{1,Collum});

% Fills up the global value Allvaluesneeded
for JJ = 1:sizeOfArrayofCoordinates
    tempx = Allvaluesneeded {Collum};
    tempx{sizeOfOveralltablex + JJ,1} = ArrayofCoordinatesx(JJ);
    tempx{sizeOfOveralltablex + JJ,2} = ArrayofCoordinatesy(JJ);
    Allvaluesneeded {1,Collum} = tempx;
end
end
```

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```
function Print_heatmap_grouplevel()
global Allvaluesneeded
% Sorting and IO for the creation of the heatmaps on a group level

OBL = 0;
OBR = 0;
OBT = 0;
OBB = 0;
VariableList = zeros(21);
VariableList = ["DeepHypothermia_trial1";"DeepHypothermia_trial9";...
   "DeepHypothermia_trial17";"DeepHypothermia_trial25";...
   "DeepHypothermia_trial33";"DeepHypothermia_trial41";...
   "DeepHypothermia_trial42";"IntermediateHypothermia_trial1";...

  "IntermediateHypothermia_trial9";"IntermediateHypothermia_trial17";...

  "IntermediateHypothermia_trial25";"IntermediateHypothermia_trial33";...

  "IntermediateHypothermia_trial41";"IntermediateHypothermia_trial42";...

  "Normothermia_trial1";"Normothermia_trial9";"Normothermia_trial17";...
   "Normothermia_trial25";"Normothermia_trial33";...
   "Normothermia_trial41";"Normothermia_trial42"];
OBL = 105;
OBR = 536;
OBT = 54;
OBB = 479;
foldername = sprintf('%s%s','Figures_for_me20/GroupHeatmaps');

%checks if the folder exists and creates a new if not
if ~exist(foldername,'dir')
    status = mkdir('Figures_for_me20/GroupHeatmaps');
end

HeatMapMax = 1.4;
HeatMapMin = 0;

%Creates heatmap and stores it at given location
for i = 1:length(VariableList)
    N = cell2mat(Allvaluesneeded{1,i});
    if ~isempty(N)
        TempHeatmap = Create_heatmap(N(:,1), N(:,2), OBL, OBR, OBB,
 OBT,...
            HeatMapMin, HeatMapMax, VariableList(i));
        NameFigure = sprintf('%s%s%s%s','Figures_for_me20/
GroupHeatmaps/',...
            VariableList(i),'_Heatmap.','jpg');
        exportgraphics(gcf,NameFigure);
        close;
    end
end
end
```

```
% Printing procedure adapted by Håvar Marsteen from Neuropragmatist
% solution posted on MATLAB answers, 9 september 2019.
% URL: "https://se.mathworks.com/matlabcentral/
% answers/479163-how-to-plot-a-position-heatmap-inside-a-circle"

function [im] = Create_heatmap(x_walk, y_walk, edgeL, edgeR,...
    edgeB, edgeT,MinVal,MaxVal,FigureName)
% counts the positions in each bin and normalizes for length of trial
Limits = [MinVal MaxVal];
nBins_x = 20; %specify bins
nBins_y = 20;
x_walk = (x_walk-edgeL);%moves the plot to the y-axis
% moves the plot to the x-axis, and rotates 90 degrees clockwise
y_walk = -(y_walk-edgeT);
stepX = (edgeR-edgeL)/(nBins_x-3);
stepY = (edgeB-edgeT)/(nBins_y-3);
x_edges = -stepX:stepX:(edgeR-edgeL)+stepX;
y_edges = -(-edgeT+edgeB+stepY):stepY:stepY;
edges = {y_edges(:),x_edges(:)};
% x y coordinates of the animal from the data,
% pluss second part of clockwise rotation
Data=[y_walk x_walk];
[counts, bin_centers] = hist3(Data, 'Edges', edges); %creating
 histogram
H = size(x_walk,1);
% normalization step, creates ratio
counts = counts./H;
% normalization step, converts ratio to seconds,
% multiplies with trial length in seconds
counts = counts.*59;
x_bin_centers = bin_centers{1};
y_bin_centers = bin_centers{2};
img = counts;
xsize = size(img,2);
ysize = size(img,1);
cent = size(img)/2;
radius = xsize/2;

% generate a circular mask
[cc,rr] = meshgrid(1:xsize, 1:ysize); % x,y indices of all map pixels
% cicular mask based on distance from cent
circ_mask = sqrt((rr - cent(1)).^2 + (cc - cent(2)).^2) <= radius;
img_masked = img; % duplicate input image
% mask everything further than radius away from cent is NaN
img_masked(~circ_mask(:)) = NaN;

FigureName = replace(FigureName,"_"," ");
figure('Name',FigureName,'visible','OFF');

% prints the bins using a linear scale between Limits
im = pcolor(y_bin_centers,x_bin_centers,img_masked);
set(im,'alphadata',~isnan(img_masked));
```

```
colormap(jet)
caxis (Limits);
axis xy tight
axis equal
axis off
title(FigureName,'FontSize', 20);
c = colorbar;
ylabel(c,'Time in bin (s/bin)','FontSize', 16);
daspect([1 1 1])
shading(gca, 'interp'); %smooths out the heat map
end
```

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