DISSECTING ENGRAMS IN NORMAL AND RECALL BASED LEARNING

Daniela Tavares Domingues

Integrated Master in Bioengineering

Molecular Biotechnology

Supervisor: Tobias Karlsson

Co-supervisor: Vasco Galhardo

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Karolinska Institutet

U.PORTO

FEUP FACULDADE DE ENGENHARIA UNIVERSIDADE DO PORTO

U.PORTO

INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR UNIVERSIDADE DO PORTO

RESUMO

Estratégias de aprendizagem baseadas no acto de recordar (método recall) têm vindo a ser apontadas como uma medida de melhorar a aprendizagem e, consequentemente, a força da memória a longo prazo. Contudo, pouco se sabe sobre os engramas subjacentes envolvidos.

Com este projeto tese pretendo estudar as diferenças comportamentais e estruturais entre métodos de aprendizagem normal e abordagens baseadas no método recall. Com este intuito, ratinhos cFos/tdTomato foram submetidos a uma variação do teste Morris Water Maze (MWM) e a sua memória espacial foi avaliada vários dias mais tarde.

Formulou-se a hipótese de que os animais do grupo recall terão uma melhor memória acerca da localização da plataforma quando em comparação com os do grupo submetidos à versão normal do teste. Para além disso, é esperado que parte dos neurónios ativos na codificação se correlacionem com os neurónios associados ao teste tardio sem a plataforma. A primeira questão foi respondida com recurso a dados comportamentais. A última usando uma linha de ratinhos cFos/tdTomato, acessível por injeção de uma droga, em combinação com resultados de immunohistoquímica contra o gene inicial imediato cFos.

Os resultados desta tese demonstram que o melhor momento para a injeção da droga ocorre aos 90 minutos depois do primeiro teste no quinto dia da experiência. Além disso, mostrou-se também que é preferível usar a versão 4-hidroxitamoxifeno em vez do comummente utilizado tamoxifeno. É ainda sugerido que as memórias desvanecem significativamente 30 a 40 dias após o teste. Por conseguinte, é este o momento apropriado para procurar melhorias na retenção de memórias a longo prazo.

A experiência final foi comprometida por um disparo inesperado do alarme de incêndio. Todavia, os resultados indicam que os engramas dos neurónios reativados dependem do cortex pré-frontal e ainda que o número de neurónios positivos se correlaciona com o desempenho comportamental dos ratinhos.

Estudos futuros estão a ser conduzidos com o objetivo de reproduzir esta experiência promissora. Este projeto procura intuir sobre as vantagens das abordagens de aprendizagem baseadas no acto de recordar (recall), propondo um novo método para as estudar em ratinhos. De facto, com o aprofundamento da investigação nesta matéria, será possível redesenhar atividades educativas, tornando a memorização num processo mais simples

ABSTRACT

Recall based learning strategies have been pointed out as a measure to enhance learning and, consequently, the strength of long-lasting memories. Nevertheless, little is known about the underlying engrams involved.

With my project thesis I aim to understand the behavioural and structural differences between normal and recall based learning approaches. In order to do this, cFos/tdTomato mice were submitted to a variation of the Morris Water Maze (MWM) task and their spatial memory was evaluated several days later.

It is hypothesized that animals in the recall group will have a better memory of the location of the target platform when compared to the normal group. Moreover, it is expected that part of the neurons activated at the time of encoding will correlate with the ones assessed in the delayed probe trial. The first question was answered by behavioural data and the last by using a transgenic cFos/tdTomato positive mice line, assessed by a drug injection, in combination with results from histochemistry staining against the immediate early gene cFos.

Results of this thesis show that 90 min after the first trial at the last day of the learning part of the experiment is the best timepoint for giving the animals the drug injection. Furthermore, 4-hydroxytamoxifen (4-OHT) is the preferable form of the drug when compared to the commonly used tamoxifen. Moreover, it is suggested that memories fade significantly after 30-40 days after the training. Hence this is an appropriate time to look for improvements in long term retention of a memory.

The final experiment was compromised by an unexpected fire alarm. Nevertheless, results point out that engrams of re-activated neurons rely on the prefrontal cortex and that the amount of tdTomato positive neurons correlate with mice behavioural performance.

Future studies are being conducted in order to replicate this promising experiment.

This project aims to give insights on the advantages of recall based learning approaches and proposes a new way of studying them in mice. In fact, with more research on this topic it will be possible to redesign certain educational activities so that remembering would be easier.

LIST OF ABBREVIATIONS

4-OHT	4-hydroxytamoxifen
°C	degree Celsius
CA	Cornus Ammonis
DAPI	4',6-diamidino-2-phenylindole
DG	dentate gyrus
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
cm	centimetre(s)
CNS	central nervous system
E	east
EC	entorhinal cortex
e.g.	for example
FA	formaldehyde
GFP	green fluorescent protein
h	hour(s)
HC	homecage control
HF	hippocampal formation
H.M.	Henry Molaison
i.e.	that is
IEG(s)	immediate early gene(s)
i.p.	intra peritoneal
I.Q.	intelligence quotient
LEA	lateral entorhinal cortex
LM	light microscope
MEA	medial entorhinal cortex
mg	miligram(s)
min	minute(s)
mL	mililiter(s)
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
MTL	medial temporal lobe
MTM	multiple trace model
MWM	morris water maze
N	north
NE	north-east
nm	nanometer(s)
PaS	parasubiculum
PBS	phosphate buffered saline

PER	perirhinal cortex
PHR	parahippocampal region
PFC	pre-frontal cortex
PNS	peripheral nervous system
POR	postrhinal cortex
PRPs	plasticity-related protein(s)
PrS	presubiculum
RNA	ribonucleic acid
RTFs	regulatory transcription factor(s)
S	south
s	second(s)
SEM	standard error of the mean
SPC	synaptic tagging and capture
SPWR	sharp wave-ripple(s)
Sub	subiculum
tdTomato	tandem dimeric Tomato
TM	tamoxifen
TRAP	targeted recombination in active populations
µm	micrometer(s)
vs	versus
W	west

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1. INTRODUCTION

1.1. MEMORY

One of the oldest quests in human history has been to understand our memory. More than 2500 years ago, philosophers were already digging into this subject. Memory was thought to be a foreign entity or an innate property of the human mind, among other theories. However, all of this discussion was being raised with a different scientific background. Only by the mid of the 19th century and with the pioneer discoveries of Paul Broca and Carl Wernicke was the brain considered not as a whole but instead possible to be divided in different functional areas (Queenan, Ryan, Gazzaniga, & Gallistel, 2017). At the beginning of the 20th century, Richard Semon invented the term "engram" and proposed for the first time that memory should be seen as a physical entity, possible to be traced (Semon, 1921). Donald Hebb then postulated that neurons that "fire together, wire together" and suggested that information might be encoded in neuronal ensembles, i.e., groups of neurons that can be recruited together and activated synchronously through synaptic connections between them (Attneave, B., & Hebb, 1950).

Karl Lashley searched for the location of memory by removing part of the cortex in rats that had learned a maze. He could only conclude that larger lesions produced more severe learning impairments and surprisingly that the location did not seem to matter. However, the task he used could easily be solved using procedural memory which might explain part of the problem (Lashley, 1950).

More insight was later gained from loss of function studies due to brain damage. One of the most famous memory research was that of Henry Molaison (H.M.). Due to his severe epilepsy, patient H.M. underwent a radical bilateral medial temporal-lobe resection in which portions of his hippocampus, dentate gyrus, subiculum, amygdala and neighbouring areas such as the parahippocampal, the entorhinal and the perirhinal cortices were removed. Following the surgery H.M's early memories and technical skills were still intact and his I.Q. was not affected (even measured as slightly higher following the procedure). Strikingly H.M. completely lost the ability to form new descriptive memories and had a retrograde amnesia spanning a few years. Consequently, the medial temporal lobe was put in the spotlight as the key area in long-term memory formation (Scoville & Milner, 1957) (Squire, 2009).

The fact that H.M. still had the ability to learn some memories albeit not descriptive indicated that different kind of learning required different brain regions. Further reports both in humans and animals solidified the view that different memory systems utilize distinct brain regions.

1.2. ENGRAMS

The term engram was formulated by the zoologist Richard Semon at the beginning of the 20th century in his Memory Engram Theory. He speculated that experience or learning new information activates and induces persistent physical and/or chemical changes in a small ensemble of brain cells. Later, if a similar stimulus is given, the same cells can be reactivated and consequently the memory involved retrieved (Semon, 1921).

Despite being a concept widely used in recent studies, the physical and chemical changes that are elicited by learning and underlie engram storage, retrieval and updating are not yet completely understood. The latest candidates for lasting memories tagging go from epigenetic alterations such as histone acetylation and DNA methylation (Korzus, Rosenfeld, & Mayford, 2004) to hippocampal sharp wave-ripples (SPW-Rs) (Buzsáki, 2015). In addition, molecular biological strategies identified a specific group of genes that are also good candidates since they are naturally expressed immediately after neuronal activation – the immediate early genes (IEGs).

IMMEDIATE EARLY GENES (IEGS)

IEGs are a specific group of genes able to respond very quickly and transiently to both cell-extrinsic and cell-intrinsic signals. They can be activated and transcribed within minutes after stimulation in a protein synthesis independent process since all the proteins required for their synthesis are already available in the cell (Bahrami & Drablos, 2016).

These genes encode a wide variety of functional proteins such as signalling molecules, transcription factors or structural proteins (Okuno, 2011). They can control the transcription of other genes or directly influence cell functioning being, therefore, classified as regulatory transcription factors (RTFs) or effector IEGs, respectively (Kubik, Miyashita, & Guzowski, 2007).

STABILITY OF THE ENGRAM

It has been shown in an associative memory study that localization of a stable neuronal ensemble correlates with memory. This means that learning and retrieval of a memory activates, in part, the same neurons. In turn, these neurons might represent the underlying engram of that specific memory. Moreover, the reactivated neurons correlate positively with memory strength (Reijmers et al., 2007).

1.3. MEMORY SYSTEMS

Memory can be divided into different domains. In relation to its durability, memories can be split into two vast groups, namely working and long-term memories. The latter, can be considered declarative or non-declarative depending on the use or not of consciousness, respectively. Moreover, depending on the type of information encoded and consequently on the brain regions involved, memory can be sub-divided into different categories. In this project I focus mainly on spatial memory that falls within the episodic-like memory type and I used the definition presented by (Squire, Genzel, Wixted, & Morris, 2015) (Figure 1).

WORKING MEMORY

In cognitive neuroscience, the term working memory replaced the more common designation short-term memory (Kandel, Dudai, & Mayford, 2014). Working memory refers to the capacity of the brain to maintain a certain amount of information over a short period of time while it is being actively processed. The precise retention interval cannot be defined but this type of memory is characterized by its limit capacity and the requirement of attention. When the capacity of working memory is exceeded, long-term memory is needed (Jeneson & Squire, 2011).

LONG-TERM MEMORY

Long-term memory refers to information that can be stored for a long period of time in the brain. In contrary to short-term forms of plasticity, RNA and protein synthesis are a compulsory requirement for lasting memories formation (Redondo & Morris, 2011).

In general, long-term memories can be divided in to two big sections, notably declarative/explicit memory and non-declarative/implicit memory (Figure 1). Non-declarative memory refers to the collection of memories that affect our behaviour even though we cannot explain exactly what is that we know. In this definition this findings include skills and habits, priming (Squire & Dede, 2015), simple forms of conditioning and reflexes (Alvarez & Squire, 1994). Declarative memory is deeply dependent on conscious memory content. This type of memory can be divided into semantic and episodic memory. Semantic memory relates to facts about the world whereas episodic memory relies on the ability to re-experience a specific event in its original context (Schacter, Eich, & Tulving, 1978). As declarative memory provides a way to represent the external world, it is often pointed out as the main guide to behaviour.

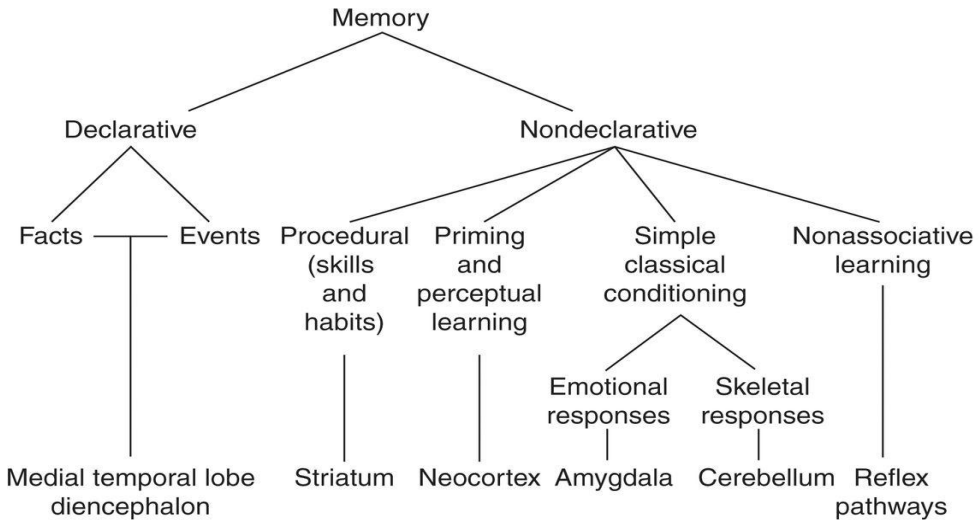

Figure 1. Organization of the mammalian long-term memory system. The figure lists the brain structures thought to be especially important for each form of memory (Squire & Dede, 2015). Declarative memory can be sub-divided into semantic and episodic memory regarding facts and events, respectively.

1.4. MEMORY STAGES

There are three main processing stages involved in memory: encoding, consolidation and re-consolidation. Encoding is the process by which memories are created. Then, memories can be stored, i.e., maintained over time and strengthen in a process called consolidation. At last, retrieval occurs when a consolidated memory is accessed and reactivated, allowing re-consolidation to happen (Banich & Compton, 2011).

ENCODING

There are two brain regions that have a crucial action in encoding: the hippocampus and the neocortex. Despite having different specializations, these two interactive systems have both a crucial role in encoding information. On one hand, the hippocampus is associated with rapid and automatic encoding, extremely necessary for acquiring information that uniquely identifies a certain event. On the other hand, the neocortex is involved in the general overlapping representations of information and therefore it has a key role on integrating new data with the existing one. All in all, the hippocampus is important to avoid interference across memories whereas the neocortex is essential to encode the main framework shared across many different experiences (O'Reilly & Norman, 2002). Furthermore, neuroimaging studies revealed a direct correlation between the amount of hippocampal activity at the time of encoding and the strength of the memory later on (Banich & Compton, 2011).

MEMORY CONSOLIDATION

Memory consolidation is the process by which a newly created, temporary memory is transformed into a more stable, long-lasting form. It can be divided into synaptic and system consolidation regarding individual neurons and the network between hippocampal and neocortical neurons, respectively (Redondo & Morris, 2011).

Synaptic consolidation refers to the cellular component of the process of consolidation. This includes the synaptic strength and the connections between individual neurons (Squire et al., 2015). The synaptic tagging and capture hypothesis (SPC) states that at the time of encoding, synapses in which synaptic plasticity occur, go through local molecular changes due to local protein translation. This is specific for the location around the active spine and serve as a "synaptic tag". This process is considered to be the trigger for the potential formation of a long-term memory. Along with the synaptic tag setting, neural activity induces the synthesis and distribution of plasticity-related proteins (PRPs). It is the capture of these proteins by tagged synapses that leads to the stabilization of the synaptic strength. Without this protein synthesis-depend phase memories cannot become stable (Redondo & Morris, 2011) Although the identity of the specific genes that encode these PRPs remains to determine (Minatohara, Akiyoshi, & Okuno, 2016), it is known that IEGs are implicated as a starting point for the signal cascade that ultimately results in local translation and the formation of a neuronal engram.

Systems consolidation refers to the overall picture of the brain regions involved in the process of storage a memory as the time passes. Two models exist: the standard and the multiple trace model (MTM).

The standard model separates memories and brain regions involved in two parts. Initially, short-term memories are formed and stored in the hippocampus. The hippocampal circuit will serve as an "index" to bind together the different neocortical sites representing the distinct parts of an episode. Later, with time, these representations become gradually dependent only on the neocortex and are considered long-term memories. Additionally, this model perceives consolidation as a time-dependent process that is influenced by in which extent new information can be related to prior knowledge (Squire et al., 2015) and by the number of repetitions involved (Born & Wilhelm, 2012). If new insights are consistent with the pre-existing learning, a similar hippocampal-neocortical-binding system takes place. Neurons are already connected together in networks called "schemas" and, therefore, this process is faster (Squire et al., 2015). On the other hand, it is suggested that repeated reactivation of temporary fresh memory representations leads to a gradually increase in complexity, distribution and connectivity among multiple neocortical networks (Born & Wilhelm, 2012) (Figure 2).

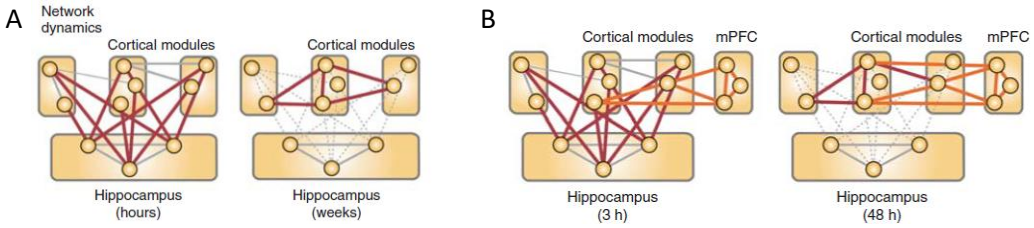

Fig.2. Standard model of hippocampal–neocortical interactions during memory consolidation. A. At the time of encoding information is stored simultaneously in the hippocampus and in multiple cortical modules. Later, these modules are gradually bound together and became independent of the hippocampus. This process is considered to be slow. B. In situations in which previous knowledge is available, there are already cortical modules connected together, independent of the hippocampus, at the start of learning. Thus, this process is quicker as involves the assimilation of new information into existing "schemas" (Born & Wilhelm, 2012).

According to the MTM, both the hippocampus and the neocortex are crucial in the processes of encoding and consolidating memories (Squire et al., 2015). At the beginning memories are mainly dependent on the hippocampus. However, with time, there is a gradually increase in the complexity, distribution and connectivity between the multiple cortical regions involved. In this way, as the memory is being stabilized, the hippocampus becomes progressively less important and the neocortex assumes major relevance. Moreover, the multiple trace model suggests that particular details are stored in the hippocampus whereas the neocortex is critical for the general outlines. In other words, episodic memories traces remain in the hippocampus while the overlapping representation of information is stored in the neocortex.

Recent findings from Tonegawa lab allowed more insights on the time and amount of participation of each region in every memory stage. Tonegawa showed that memories are actually formed simultaneously in the hippocampus and in the long-term storage location in the cerebral cortex. In fact, at the time of learning, hippocampus-cortex circuits are engaged rapidly and together. However, despite being immediately active, engrams in the hippocampus gradually fade into a "silent" state. On the other hand, the "silent" memories in the cortex need to undergo through anatomical and physiological changes during two weeks to reach a mature state (Kitamura et al., 2017). Overall, these results suggest consolidation as a complementary memory system where the hippocampus allows rapid active memory formation but has limited capacity and the neocortex retains longer-lasting information but its cortical engrams need time to develop. Moreover, it was shown that this communication is so important that just blocking the circuit connecting those two regions prevented the cortical memory cells from maturing properly. Nevertheless, further studies are needed to determine whether memories fade completely from hippocampal cells or if some traces remain. Also, it is known that hippocampus represents spatial information using "place" cells that map the environment but it is not clear the contribution of the prefrontal cortex (PFC) in this process (Eichenbaum, 2000).

MEMORY RECONSOLIDATION

The state of long-term memories is not permanent. In fact, it involves a dynamic process named memory reconsolidation that allows memories to be modulated, i.e., weakened or enhanced. When memories are retrieved, i.e., when they are actively recalled, consolidated memories can revert to a vulnerable state. With this destabilization they can undergo another consolidation process, similar to that of a new memory, and be disrupted or associated to parallel traces (Alberini & LeDoux, 2013).

Also in this stage, the major role is played by the hippocampus and the prefrontal cortex. But in addition it is also noteworthy the involvement of the left parietal cortex in remembering old items. Moreover, being memory a highly interactive system, it is no surprise that the amount of activation observed in the PFC is closely related with the encoding phase since poor encoding makes retrieval more difficult and proper encoding facilitates the process (Ribeiro & Nicolelis, 2004).

1.5. RECALL-BASED LEARNING

Until not so long ago, studying was seen as the key process to promote learning and on the other hand testing was believed to be a neutral way of assessing knowledge (Roediger & Butler, 2011). Nevertheless, at the end of the 20th century, Endel Tulving highlighted the importance of memory and retrieval in a distinctive way, placing the absence of information and the lack of recalling it at the same level. "If you know something or if you have stored information about an event from the distant past, and never use that information, never think of it, your brain is functionally equivalent to that of an otherwise identical brain that does not "contain" that information" (Karpicke, Blunt, & Smith, 2016).

Retrieval is the active act of reconstructing knowledge from past experience and the most common way of assessing it is by tests. In the last decades Karpicke and others have dedicated most of their research to study the outcomes of recall-based learning, i.e., the effects of retrieval practices in the learning period on the memory strength.

Retrieval has been shown to enhance the process of learning both in adolescents and children and in a wide variety of materials such as lists of words (Karpicke & Roediger, 2007) (Karpicke, 2008), scientific materials (Karpicke & Blunt, 2011), vocabulary (Goossens, Camp, Verkoeijen, & Tabbers, 2014) or word pairs (Goh & Lu, 2012). Yet, most of the research subjects surveyed, mainly students, did not expect this outcome and devalue the actual benefits of it (Karpicke et al., 2016).

For these reasons, certain educational activities should be redesigned so that remembering would be easier and information about the advantages of retrieval practices should reach the public at large. However, while the behavioural effects of this type of learning are clear, there is still a lack of knowledge of how recall based learning differs in the way engrams are created or how it affects their stabilization.

1.6. BRAIN REGIONS

The cognitive memory system relies essentially on three major systems namely the medial temporal lobe, the temporal cortex and the frontal cortex. More precisely, neural circuits in the medial temporal lobe are involved in the association between environmental stimuli and behavioural contexts (the basis for episodic memory); the ones in the temporal cortex represent repeated associations (the basis for semantic memory) and the frontal cortex is crucial for active retrieval (Miyashita, 2004).

As the core of this project is to understand the engrams underlying spatial memories and the act of retrieving them, mainly the hippocampus and the pre-frontal cortex are going to be analysed.

HIPPOCAMPUS

Within the temporal lobe, especially in the medial region, there are a set of organized structures called the hippocampal formation. The hippocampal formation is constituted by a three and a six layered area. The hippocampus proper, the dentate gyrus and the subiculum belong to the three layered division whereas the presubiculum and the parasubiculum are part of the six layered division.

The hippocampus proper is also named the Ammon's horn (Cornus Ammonis) after the Egyptian god Ammon. This is due to its resemblance in crossections with that god's appearance - a human body with a head of a ram with a horn. The hippocampus proper can be divided into CA1, CA2 and CA3 based on its pyramidal cell density (Radonjic et al., 2014).

Many of the hippocampal formation connections with other brain regions are unidirectional. Basically, the dentate gyrus receives its inputs from the entorhinal cortex (EC), especially from cells located in layer II, via fibers called the perforant path. The dentate gyrus projects to CA3, CA2, CA1 and the subiculum. The hippocampal formation efferents can reach not only cortical but also subcortical regions (Van Strien, Cappaert, & Witter, 2009) (Figure 3).

Within the hippocampus, the dentate gyrus has been pointed out as a crucial region for three major mechanisms: conjunctive encoding of multiple sensory inputs, pattern separation and temporal integration (Kesner, 2013). Its role in memory is crucial so this region has received a lot of attention. Moreover, this is one of the easiest identifiable sub-regions of the brain in the microscope.

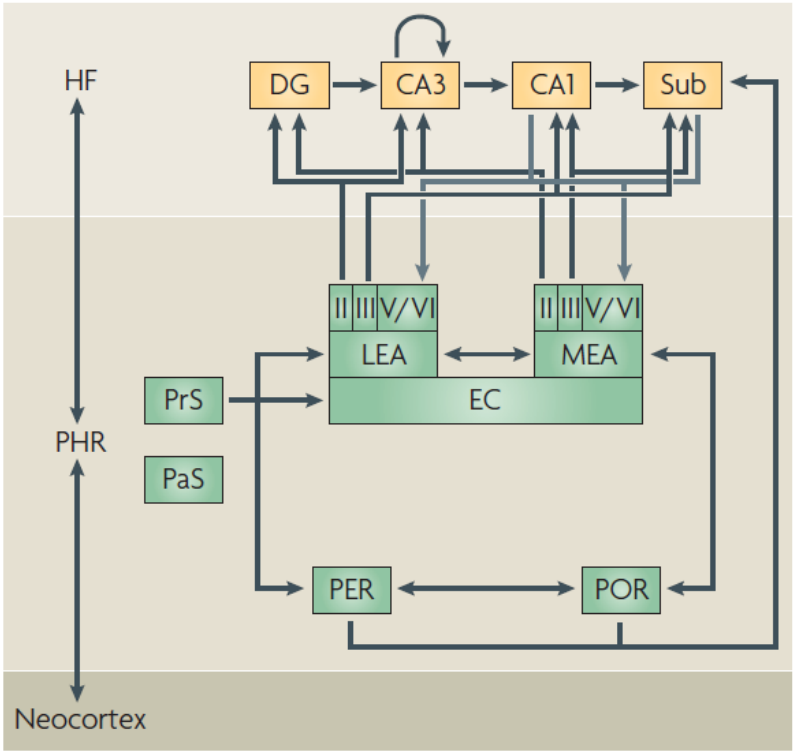

Fig. 3 Neuronal circuit between the hippocampal formation (HF) the parahippocampal formation (PHR) and the neocortex. DG: dentate gyrus; EC: entorhinal cortex; HF: hippocampal formation; LEA: lateral entorhinal cortex; MEA: medial entorhinal cortex; PaS: parasubiculum; PER: perirhinal cortex; PHR: parahippocampal region; POR: postrhinal cortex; PrS: presubiculum; Sub: subiculum; (Van Strien et al., 2009)

PRE-FRONTAL CORTEX (PFC)

Within the frontal lobe, the pre-frontal cortex can be sub-divided according to its different functions, namely the anterior part is involved in planning and the posterior one in movement; the medial part is associated with emotion whereas the lateral one with logic, being the ventrolateral region a major contribute to selection of goal-relevant item information and the dorsolateral important for the organization of multiple pieces of information in working memory (Blumenfeld & Ranganath, 2007).

1.7. BEHAVIOURAL PARADIGM

In order to study the main question of this project, a spatial task, more precisely, a variation of the Morris Water Maze, was chosen as the behavioural paradigm.

When performing spatial tasks animals can rely on two different types of navigation: allocentric and egocentric. When using allocentric navigation, mice rely on distal cues referred to as landmarks. These landmarks are located farther away from the organism and provide a relative position of the animal to its surroundings. Egocentric navigation, involves internal cues such as rate of movement, sequence of turns or signposts. Signposts differ from landmarks because they are close and hence only a marker of where to change direction along a path; they do not provide any type of relational information whereas landmarks do. (C. V. Vorhees & Williams, 2014)

The primary brain regions that mediate these two types of navigation overlap but at some extent can also be distinct from each other. Although less well studied, it is known that the dorsal striatum and its connected structures are essential for egocentric navigation. Moreover, the cerebellum is involved in procedural aspects of the task. On the other side, loss of function studies revealed that allocentric wayfinding is impaired when lesions are located in the hippocampus, the entorhinal cortex or the surrounding structures. In fact, the role of the hippocampus appears to depend upon its reciprocal connections with the ento- and perirhinal cortices. The brain can switch between using these two different systems to solve a task. A task that can be performed often and in a very similar manner either rely on allocentric or egocentric navigation. If the process becomes automated, it can turn into a habit. When this occurs, the location of the memory shifts within the brain and is reclassified as an implicit procedural memory. (C. V. Vorhees & Williams, 2014) (D'Hooge & De Deyn, 2001).

Overall, spatial learning constitutes a functionally integrated neural network where the coordinated action of different brain regions and systems are crucial to good functioning. Nevertheless, little is known about the specific systems that are involved in a specific engram.

MORRIS WATER MAZE TEST

The Morris Water Maze (MWM) is a well-established test of spatial learning for rodents developed by Richard Morris in 1981 (Morris, 1981). In this task, rodents are placed into a large circular tank from which they can only escape by finding a hidden platform. This platform is "invisible" because it is beneath the surface of an opaque liquid (eg.: water and a nontoxic white paint or milk). In this way, the animals can only escape if they learn, across repeated trials, the spatial position of the platform relative to distal cues by landmarks association.

In order to do this, animals rely on allocentric navigation and therefore, episodic rather than procedural memory. To ensure this, the platform is always in the same position but the rodents start swimming from different locations and need to actively search for it. Once the mice have reached the platform they remain there for 30 seconds before the trial is considered to be completed. Those 30 seconds allow the mice to investigate the landmarks and their relative positioning to the platform. If the animal fails to find the platform within the 90 s trial period, it is lifted to the platform and allowed to stay for 30 s on it to facilitate the process of learning its location. Across repeated trials, the rodents learn to move efficiently and directly to the platform.

Twenty-four hours after the last acquisition session test, animals are placed in the pool without the platform there, a so called probe trial. Here they are given 60 s (a longer time could result in them giving up) to search for the platform's former location. If the animal has learned it, it should show a preference for the quadrant in which the platform was located and even for the site where the platform was within the target quadrant. Proximity to the platform site, i.e., average distance to the platform, is one of the best measurements to address the amount of learning that has occurred (Maei, Zaslavsky, Teixeira, & Frankland, 2009).

ADVANTAGES

Ease of testing The basic procedure is relatively simple and it is quite easy to have the perfect apparatus – an uniform pool without proximal cues, located in a room with different surrounding landmarks. In addition, due to the movement of the water in the water maze, there is reduced likelihood of odour trails interfering with learning and there is little training required to obtain high levels of proficiency.

Motivation Mice are natural swimmers but still prefer to be out of water. Water is not only a sufficient incentive but also an equal-opportunity motivator. Indeed, nearly all animals complete the task which avoids the problem of selection bias and guarantees rapid and reliable learning with little training required. Moreover, their motivation does not depend on differences in body weight, appetite or the reward value for the reinforcement. In fact, water is as motivating on the last trial as it is on the first.

Animals difference We use several control variables to ensure that any difference between groups are not caused by a behavioural effect. The system we use records a large set of parameters such as, for example, swimming speed and thigmotaxis. These indicators are always compared between the groups and non- and bad performers that develop floating or thigmotaxis behaviour are removed from the study. Nevertheless, animals can be under stress and that circumstance, even if more difficult to identity, should also be taken into consideration. For instance, animals should be handled before the experiment starts.

CONSIDERATIONS

The Morris Water Maze is influenced by many factors such as the characteristics of the experimental animals as well as by the apparatus and the training procedures.

Animals Regarding the object of study, differences exist depending on the species/strain, sex, age, among other characteristics of the animals. By way of example, mice perform worse than rats in swimming tasks – their floating and/or thigmotaxis behaviour tend to be more pronounced. Also, male animals tend to perform better than females. Although this difference is almost inexistent if the females are in their estrus phase or if the animals are young (up to 6 months). It is also important to consider the nutritional status of the animal (D'Hooge & De Deyn, 2001) as well as their levels of stress (Kim, Lee, Han, & Packard, 2001). It is possible to try to minimize their stress by handling them a few days before the start of the experiment (Holscher, 1999) and by placing them in the behaviour room in advance. However, this type of factors are difficult to control so, as in all behavioural studies, it is critical that all the animals pass through the same exact conditions. Furthermore, one of the major concerns when working with animal research is the 3 Rs (reduce, refine and replace) policy. In this regard, it must not be forgotten that underpower experiments cost more time, more money and more animals to prove or disprove the presence of false positives. (Charles V Vorhees & Williams, 2006)

Apparatus Initially, the Morris Water Maze was developed as a spatial task for rats. Later, modifications mainly on its set up enable an adapted version for mice. Overall, it is important to consider the alterations made, namely the size of the apparatus, the temperature of the water and the intertrial intervals duration. With regard to the test setup, the pool and the platform can't be too small or too large to prevent the animals to solve the task without using spatial cues or conversely give up on the test. In relation to the water temperature, both water too cold (Rauch, Welch, & Gallego, 1989b) or too hot (Rauch, Welch, & Gallego, 1989a) impair MWM learning due to hypotermic and reduced motivation, respectively. So, water at room temperature, typically around 19°C - 22°C, have been found to be at the optimal range. Also related with the animals temperature is the intertrial intervals duration. It has been shown that animals that have longer intertrial platforms (10 or more minutes) perform significantly better than the ones that do not have them (Commins, Cunningham, Harvey, & Walsh, 2003).

Training procedures To obtain good learning, the Morris Water Maze procedure should involve four trials per day for 4-7 days with a limit per trial of 90-120 seconds or 60-90 seconds without the platform.

SIGNIFICANCE

Besides being a spatial test for rodents, the MWM assesses a conserved type of learning that is common to both animals and humans due to its parallel importance for survival. Moreover, most of the properties that affect animals' performance are the same in humans. For example, stress (Holscher, 1999) and aging (Sauro, Jorgensen, & Pedlow, 2003) are two major factors that impair navigation tasks in both species.

In a virtual navigation task, human participants encountered a maze that could be solved either in a spatial or a non-spatial way. At the outset, participants who used a spatial approach showed increased activity in the right hippocampus whereas the non-spatial strategy participants showed increased activity in the caudate nucleus, a striatum structure (Iaria, Petrides, Dagher, Pike, & Bohbot, 2003). This result shows that, in fact, the same network that provides spatial information in rodents is implicated in humans since the same brain regions are involved. In a different study, an adaptation of the Morris Water Maze to humans by virtual reality revealed no significant differences between human and rodent performance. This shows, again, the general validity and the implied cross-species comparability of this test (Schoenfeld, Schiffelholz, Beyer, Leplow, & Foreman, 2017).

All in all, the Morris Water Maze is a test that allows research on semantic and episodic memories. In humans, this research gains even more importance since it allows the study of the failure of the systems involved, quite common in certain diseases and injuries such as, for example, the Alzheimer (Hirni, Kivisaari, Monsch, & Taylor, 2013) or the Huntington's disease (Montoya et al., 2006). Having more insight on the basic mechanisms behind these problems will enable the development of targeting treatments (C. V. Vorhees & Williams, 2014).

1.8. TARGETING NEURONAL POPULATIONS

The ultimate ambition of neuroscience is to understand the principles behind our neural networks and their connectivity, and how the nervous system processes information and drive our behaviour. In order to achieve this goal it is not sufficient to study single isolated neurons but instead the brain has to be studied and analysed also in its complete state where focus is put on its interconnections in all different levels.

Thanks to recent advances in genetic tools it is now possible to dissect neural circuits at a much more detailed level than before. Furthermore, it is now possible to establish a correlation between the activity of specific groups of neurons, persistent synaptic changes and an animal's memory-associated behaviour. However, caution must be taken when establishing this type of cause-effect relationships because the underlying mechanisms are complex and require both extremely precise genetic tools and compatible readout methods. Furthermore, the application of these tools depends critically on the ability to target them to specific subpopulations of neurons and on the criteria used to define these subpopulations.

Neurons can be pooled in different groups and thus traced depending on their cell type or morphology, anatomical location, genetic background, among others parameters. However, since neuronal populations that contribute to the same network can be developmentally, anatomically and genetically different, tracking neurons based on a functional criterion - their activation by a stimulus - is most likely a more functionally accurate way to group them.

TARGETED RECOMBINATION IN ACTIVE POPULATIONS (TRAP)

TRAP is a genetic tool which allows permanent tagging of neurons that were activated by a specific stimulus or experience.

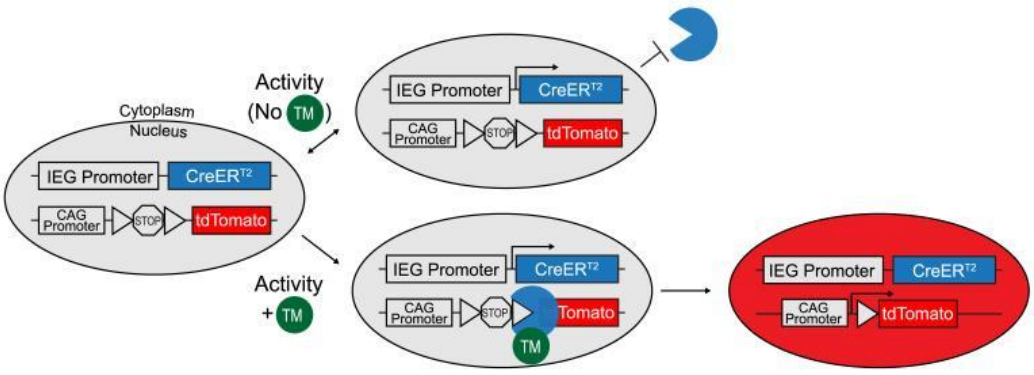

Figure 4. Strategy of TRAP. In this work, the IEG is c-Fos and the TM (tamoxifen) drug was replaced by one of its metabolized version named 4-OHT (4-hydroxytamoxifen) (Guenthner, Miyamichi, Yang, Heller, & Luo, 2013).

The method used in the proposed work is a binary expression strategy that requires two different transgenes: one that expresses a tamoxifen (TM)-dependent Cre recombinase (CreERT2) from an activity-dependent IEG promoter, in this case c-Fos; and one that allows the expression of an effector gene in a Cre-dependent manner, in this case a fluorescent molecule named tdTomato (Guenthner et al., 2013).

A c-Fos promoter is used to drive the expression of the $CreER^{T2}$.

c-Fos is an IEG so, as previously stated, it naturally turns on immediately after neuronal activity. In this way, the IEG promoter induces the expression of the downstream transgene only in neurons that are recently active. As a result, solely activity-dependent cells are label (Kubik et al., 2007). The c-Fos promoter in particular was chosen because its expression leads to good results since its background levels are low, it can be induced by different extracellular signals as well as combined with several markers and its response is transient and easy to detect (Kovács, 2008).

$CreER^{T2}$ is used in order to control site-directed recombination. This protein results from a fusion between the phage DNA recombinase Cre and a modified estrogen-binding domain of the estrogen receptor. With the administration of an estrogen analog such as tamoxifen (TM), the fusion protein translocates from the cytoplasm, where it is normally retained, to the nucleus. In the nucleus it activates recombination (Feil et al., 1996). Since its action is dependent on a third exogenous molecule, this design guarantees the construct a time-dependent faculty. In fact, without this feature, the IEG promoter would express the DNA recombinase, by default, any time the cell fired. Instead, this way, it is possible to control its time window of action.

tdTomato is a tandem-dimer version of the orange fluorescent protein Tomato. It is constituted by two chromophores linked covalently, containing the first and last seven residues from GFP on its N- and C-termini. For these reasons, this variant behaves like a monomer and is associated with high fusion proteins tolerance and low localization artefacts. It is also ideal for *in vivo* studies due to its extreme brightness, excellent photostability and low aggregation levels (Shaner, Patterson, & Davidson, 2011)

In the construct, the effector protein is under the control of the strong and ubiquitous CAG promoter, followed by a transcription stop condon flanked by two recombinase target sites, the loxP sites. In this way, the tdTomato is expressed in a recombination-dependent manner. Indeed, only cells expressing the two transgenes can have the loxP sites cut by the tamoxifen dependent DNA recombinase, the transcription stop codon delete and, consequently, the expression of tdTomato (Luo, Callaway, & Svoboda, 2008). Since this protein fills cell bodies, dendrites and axons, recombined cells can be easily identified by their morphology (Shaner et al., 2011).

4-hydroxytamoxifen (4-OHT) has been considered one of the most active metabolites of tamoxifen (Etienne et al., 1989) Tamoxifen is an estrogen analogue that binds with higher affinity than estrogen to altered estrogen binding domains. Guenthner et al., showed that direct injections of 4-OHT allow access to neuronal ensembles activated less than 12 hours after its injection. In fact, with this metabolized version, the time window in which the neurons can be labelled is way much shorter than with tamoxifen - 6 hour prior to the injection and up to 24-36 hour after the injetion - (Guenthner et al., 2013). Thus, TRAPed populations achieved by 4-OHT injections are closer to be more similar to the real neurons activated during the learning phase of the experiment. Also, as the neurons are permanently labelled, just by waiting, the fluorescent marker (in this case the tdTomato protein) can diffuse throughout the cell and, that way reveal detailed neuronal morphologies of the TRAPed populations.

All in all, with this strategy, mice submitted to a new task express learning-induced IEGs such as c-Fos which in turn are a marker for active nerve cells.

In sum, the c-Fos promoter drives the expression of the CreER^{T2}. This DNA recombinase removes the stop codon of the construct and enables the expression of tdTomato. The specific neurons activated during the 4-OHT time window are labelled for as long as they survive.

The possible underlying mechanisms are described in Table 1.

	Non-active cells	Active cells
No tamoxifen	CreER^{T2} is not expressed → no recombination occurs	CreER^{T2} is retained in the cytoplasm → no recombination occurs
Tamoxifen		CreER^{T2} is expressed → recombination occurs → expression of tdTomato

Table 1. Possible results obtained with the TRAP system (Based on (Guenthner et al., 2013).

Note that the tamoxifen lifetime is limited by its metabolism and excretion in the animals' organism and the CreER^{T2} lifetime is limited by the transient nature of the IEG transcription, in this case c-Fos. Only neurons that are active within a limited time window around drug administration can be TRAPed (Guenthner et al., 2013).

2. AIMS

2.1. MAIN AIM

The main aim of this thesis is to find a way of assessing normal and recall based learning in an animal model. The goal is to study how it affects learning and to assess the activity pattern of the neuronal ensembles between those two groups.

2.2 QUESTIONS

There are three major questions in study:

1) Is it possible to assess normal and recall based learning in mice performing a Morris Water Maze learning task?
    - If so, how stable is that memory?

2) Are the same engrams involved in learning and memory?
    - Is it possible to specifically label "learning" and "memory" neurons?
    - Are more neurons activated during the beginning or at the end of the Morris Water Maze?
    - Does the activity pattern switch to a new brain region? Do they co-localize with recently active neurons?

3) Is there any correlation between mice behavioural performance and their engrams on a cellular level?

2.3 HYPOTHESIS

It is hypothesized that mice in the recall group should remember the behavioural task better than the ones in the normal group.

It might be possible to assess the specific underlying engrams involved in the process of remembering by looking at co-localization between neurons labeled at the learning part of the experiment by the TRAP system and at the late phase by immunohistochemistry against an immediate early gene, in this case c-Fos.

3. MATERIALS AND METHODS

3.1. ANIMALS

All mice used in the different experiments were breed in the animal house at the neuroscience department of Karolinska Institutet, in Sweden. Animals were group housed separated based on sex and with 2 to 5 mice per cage. Houses were plastic-bottomed cages, containing cardboard houses and paper strips. Animals were housed in a temperature control environment (22-23°C), which was maintained on a fix 12:12 hour light-dark cycle (light from 06h00 to 18h00). All mice were given *ad libitum* access to water and food. Experimentation took place during the light phase and all subjects were handled for 30 seconds per day for 5 days before experiments began.

For the different experiments mice were grouped based on their genotype (tdTomato mice vs not tdTomato mice) and their sex (female vs male). After, mice were semi-randomly distributed into the different groups of the experiments. When possible, age and weight were also taken into consideration in order to have balanced groups.

During test days mice were placed in the behavioural room at least 1 hour before the experiment start to minimize stress effects. Control animals stayed in their homecage during the entire experiment but were subject to the same transportation and to identical time in the behavioural room. This way, their stress levels and activated neurons were closer to the baseline of the experimental groups. Mice that did not learn the behavioural task, i.e., that in the last two days did not find the platform more than 50% of the trials, were removed from the study. The specific number and other relevant information about the animals used in each experiment in particular, for e.g., their genotype is described in the results section once it differs from experiment to experiment.

Guidelines for the maintenance and experimentation of animals were followed according to Stockholm Animal Ethical Committee - ethical permit number N246/15.

c-FOS/TdTOMATO MICE

The double heterozygous mice used were obtained from Jackson labs. They result from crossbreeding $Fos^{CreER/+}$ and $R26^{AI14/+}$ mice. $Fos^{CreER/+}$ have the following construct:

Fos - $CreER^{T2}$ – deltaNeo – deltaDTA	
Fos	c-Fos promoter, an IEG promoter
$CreER^{T2}$	$CreER^{T2}$ –SV40 polyA, a Cre-recombinase cassette
deltaNeo	psV40-NeoR-polyA cassette (deleted), used for positive selection
deltaDTA	diphtheria toxin A cassette (deleted), used for negative selection

Table 2. $Fos^{CreER/+}$ construct

$R26^{AI14/+}$ mice are animals with the following construct:

Rosa-CAG-LSL-tdTomato-WPRE-deltaNeo	
Rosa	Gt(ROSA)26 locus; expressed in most tissues of the mouse and therefore in all active neurons
CAG	CMV-IE-enhancer/chicken beta-actin/rabbit beta-globin hybrid promoter; a strong synthetic promoter
LSL	loxP-flanked STOP cassette
tdTomato	fluorescent protein, stable *in vivo*
WPRE	woodchuck hepatitis virus post-transcriptional regulatory element, a mRNA transcript stability enhancer
deltaNeo	attBl attP-flanked PGK-FRT-Neo-polyA cassette (deleted), used for positive selection

Table 3. $R26^{AI14/+}$ mice construct

3.2. THE MORRIS WATER MAZE

The Morris Water Maze was chosen as the behavioural paradigm as it is a broadly studied and demanding task to assess spatial learning and memory. The water maze (1.8 m in diameter) was filled with water to a depth of 25 cm and maintained at room temperature (22-23°C). A circular escape platform (15 cm in diameter) was placed in the center of a quadrant of the tank, submerged 1 cm below the surface of the water. Several endogenous cues were available in the room, including three intentional different black and white pattern suspended cardboards (Figure 5) to allow visual navigation. Cues location was constant over the testing period in order to assist mice finding the platform. The animal´s movements for each trial were recorded by a camera positioned directly above the center of the maze. The digital tracking software Biobserve was used to collect information regarding escape latency (time from the moment the mice is placed into the pool until it finds the platform), swim distance, average target distance (average distance to the platform) and time and crossings in the platform zone.

Mice were trained for 5-7 days, with four trials per day (20-28 trials in total). In each trial, mice were placed into the water maze from one of four starting positions – north (N), east (E), south (S) and west (W) (Figure 5). During a day each mouse used a different starting position in each trial, chosen semi-randomly as each position was only used once per day and in a different order. This set up was chosen to prevent mice from using procedural memory instead of hippocampal dependent memory to solve the task.

In order not to compromise the results with hypothermia effects, in between trials mice were warm up - as the cage was in contact with a heating mat - and a 15 minutes inter trial interval was used for each individual mice.

Each trial lasted until the mice reached the target (escape latency) or a maximum of 90 seconds, after which they would be placed on the platform by the experimenter. After that, animals remained 30 seconds on the platform in order to remember its position regarding the surrounding cues. The platform was placed in the middle of what was considered the north-east quadrant of the water maze. Its central position is justified since diminishes the chance of the animal find it randomly with a thigmotaxis behaviour, i.e., by swimming at a constant distance from the wall around the pool or swimming to the center of the pool.

For assessing recall-based learning, a variation of the Morris Water Maze was used in the learning phase. In this setup mice still train for 5 or 7 days, depending on the experiments, with four trials per day. However, in one of the trials on each day mice were subjected to a probe trial - a trial in which the platform is removed. This trial lasted 60 seconds instead of 90 seconds to avoid any possibility of mice developing defeated behaviours or memory extinction. This type of trial took place during the second or third trial of the day. This type of concerns intends to avoid mice being able to do any kind of association that can lead them to give up on the task on this type of trials.

In order to assess the level of learning, mice were subjected to a probe trial twenty four hours after the training phase, regardless of the experimental group. As mentioned before this trial took place for 60 seconds and it started in one of the farthest locations from the platform. In this way, the possibility of them being close to the platform by chance is minimal.

Figure 5. Panoramic view of the behavioural room. The pool is placed in the centre of the room with 3 landmarks (triangle, square and circle) with high contrast around it. Overhead is a camera that tracks the movement of the mice.

3.3. STRATEGY

The strategy proposed in this thesis is to permanently label the neurons activated in the learning part of the experiment with a drug injection and later assess reactivated neurons by immunohistochemistry against an immediate early gene (Figure 6).

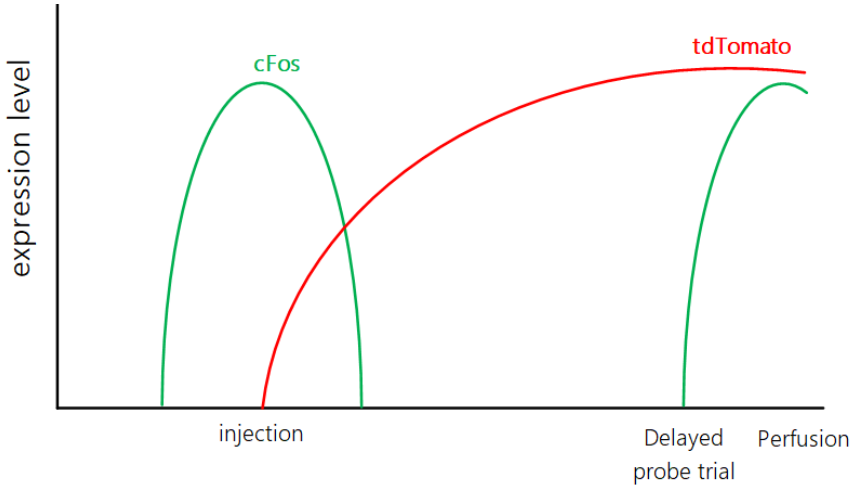

Figure 6 Schematics of the expression levels of c-Fos and tdTomato. c-Fos time window of expression are a few hours whereas tdTomato permanently labels the neurons that recently had c-Fos activation.

3.4. DRUG PREPARATION

Tamoxifen (Sigma, Cat #T5648) was dissolved at 20 mg/mL in corn oil (Sigma, Cat #C8267) as described in (Guenthner et al., 2013).

4-hydroxytamoxifen (4-OHT) (Sigma, Cat H6278) was dissolved at 20 mg/mL in ethanol absolute by shaking at 37 °C for 30 min. Corn oil (Sigma, Cat #C8267) was added to give a final concentration of 10 mg/mL 4-OHT. Later, ethanol was evaporated by vacuum centrifugation (ramp level 2, 15 torr).

Both, the final 20 mg/mL TM and the 10 mg/mL 4-OHT solutions were prepared in the same day of the injection and were delivered intraperitoneally (i.p.), 150 mg/kg of TM or 50 mg/kg of 4-OHT.

The drug preparation and the dosage here presented were based in the previously described protocol in (Guenthner et al., 2013).

3.5. TISSUE PROCESSING AND IMMUNOHISTOCHEMISTRY

Mice were anaesthetized with pentobarbital (APL, 3039275) and perfused transcardially with 10 mL of thyroid with heparin (LEO, 015226-06) (a 1:2000 mixture of heparin : thyroid solution) followed by 50 mL of 4% formaldehyde (Sigma, Cat #252549) (FA) in PBS. Brains were dissected and post-fixed for 24h in the same fixative. Thereafter, they were rinsed and kept in sucrose for at least three more days, being the sucrose solution changed every day.

For immunohistochemistry, brains were cryosectioned into 14-40 µm slices (thin sections) or 200-400 µm slices (thick sections).

Thin sections were rehydrated in PBS (10-15 min) three times and covered with blocking solution (PBS with 0.1% Triton (Sigma, Cat #93443) and 5 % goat serum) for at least 1 hour. After blocking, a rabbit anti-cFos primary antibody (1:800 Synaptic Systems, #226003) was added and sections were incubated overnight at 4° C. The following day, slides were washed in PBS and an anti-rabbit Alexa 633 (1:400 Vector, #DI-1649) fluorescent secondary antibody combined with DAPI (Invitrogen, #D1306) was added for 1 hour. Brains were then washed again and mounted using prolong (Invitrogen, #P36930). DAPI was added to every slide as a nucleic acid fluorescent staining in order to allow easier visualization of the regions of interest. Fluorescent images were taken in Karolinska BRICK Facility using a Zeiss 800 LM confocal microscope and a Zeiss 800 LM Airy scan confocal microscope for higher resolution pictures. Images were processed in ImageJ to adjust contrast and brightness of each channel and in ZEN to do measurements and counting of cells.

Thick sections were cleared following an adaptation of a previously described protocol (Ke et al., 2016). Sections were rehydrated in PBS (10-15 min) three times and then permeabilized with a 2% saponin (Sigma, Cat #84510) in PBS solution for at least 2 hours. After this step, sections were subjected to a 2% saponin solution with ultrapure water and Omnipaque350 (a contrast agent used for x-rays) (GE Healthcare, 1181332) overnight at room temperature. After that, for the following three days, sections were transfer to the same solution but with an increase percentage of Omnipaque (2:1 mixture to a 1:1 mixture).

4. RESULTS

The focus of this thesis was to study the different outcomes, both behavioural and structural, of normal and recall-based learning and how it affects lasting memories. To assess the behavioural part of the project mice performed a variation of the Morris Water Maze test (described in the previous section); to investigate the structural basis of the neural ensembles involved, tdTomato neurons were analysed.

Homecage controls are animals that never swam in the pool but spent the same amount of time in the behaviour room as the experimental groups.

For all the Morris Water Maze behavioural analysis, mice were analysed in three different phases: 1) the learning part which consisted of the first 5-7 days in which the animals were learning the task; 2) the 24h after probe trial in which the animals were put in the pool 24h after the learning period without the platform and 3) the delayed probe trial when the animals were again placed in the pool without the platform there after a delayed period of time.

In the learning part of the experiment the animals learn the behavioural task, i.e., that in order to be rescued from the pool, they need to find the platform and stay there. All animals were individually analysed per day in terms of escape latency – time until they reach the platform – and swim length. Animals that revealed thigmotaxis behaviour, i.e., swam only close to the walls; or developed a floating behaviour, i.e., that didn't even swim, instead just floated around in the pool, were excluded from the experiment. Furthermore, a learning criteria was in place so that all animals that didn't find the platform more than 50% of the times in the last two days of the learning part were excluded from the experiment.

Although the major goal of the Morris Water Maze is to assess hippocampus dependent memory, animals can solve the task using a strategy based on procedural memory, swimming at a certain distance from the wall. In order to control this and assess specificity of learning, mice performed probe trials (trials where the platform is removed). In this test the average distance from where the platform used to be (average target distance) and the amount of time they spent in the quadrant that used to contain the platform was used as learning variables.

4.1. MORRIS WATER MAZE SET UP

AIM

A pilot experiment was performed in order for me to learn to handle a big behaviour experiment and test the setup for the Morris Water Maze.

EXPERIMENTAL SET UP

From the 12 animals used in this experiment 3 were excluded because they did not fulfill the learning criteria defined previously. Out of the mice that participated in the experiment, 4 animals swam the maze for 5 days and then were sacrificed for assessing c-Fos expression in a pilot experiment (see result 4.4) and 5 swam for an extra day, in order to study their behavioural performance. The ones in the last group also performed a probe trial 24 h later. All animals were plotted in a chart and learning levels were evaluated (Figure 7A and Figure 7B). Charts regarding the learning period were re-plotted only with the animals which participated in the probe trial too (Figure 7C and Figure 7D).

RESULTS

Overall mice reduced their average escape latency over the experimental days. The learning analysed using swim length matches that of latency. At the last day of the Morris Water Maze they took around 50% of the time and swam around 50% of the length than in the beginning of the experiment (Figure 7).

In regards to the probe trial, mice were analysed taking into account the % of time they spent in the correct quadrant (NE). Kice significantly improved their performance and spend significantly more time in the target quadrant (Figure 8).

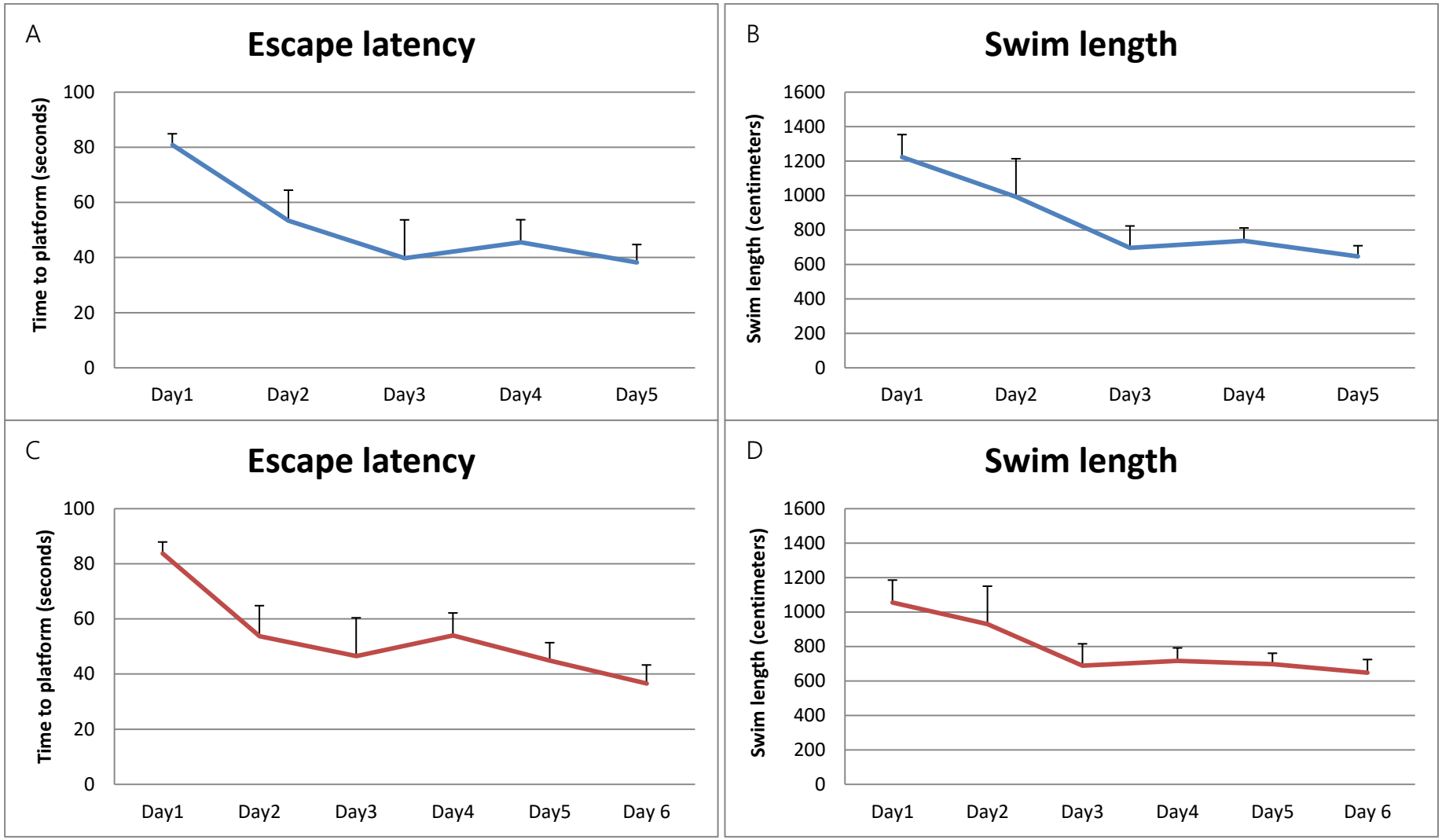

Fig. 7 A) Average escape latency and swim length (B) of all animals involved in the experiment (n=9). At day 5, 4 mice were sacrificed to investigate c-Fos expression C) Average escape latency and swim length (D) of the 5 animals that were involved in the both parts of the experiment (n=5) – the learning phase and the 24h later probe trial.

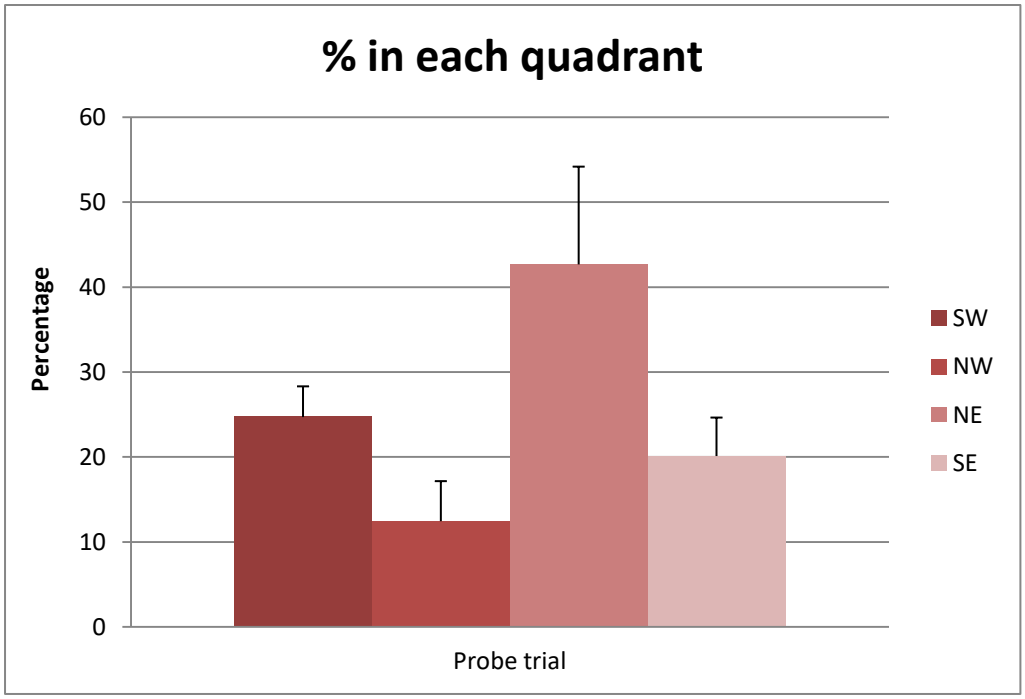

Fig. 8 Percent of time spent in each quadrant during the probe trial. Mice spent significantly more time in the quadrant that used to contain the platform (NE) (n=5).

CONCLUSION

Mice reduced their average escape latency and average swim length by 50%. However, according to the literature this decrease should be around 75%-70% and not only of 50%. This result indicates that mice did not reached optimal learning in this study. This could have happen due to several factors but in particular three can be highlighted – 1) this was my first time performing the Morris Water Maze, 2) mice were not handled before the experiment started, 3) cues might have been located too high in the behavioural room, complicating mice navigation task.

The probe trial shows that mice searched for the platform in the correct quadrant. This is a clear indicator that they learnt the location of the platform based on the quest and not by using procedural learning.

4.2. MORRIS WATER MAZE OPTIMIZATION

AIM

This experiment was performed to check if the Morris Water Maze optimal learning levels could be achieved with the suggested alterations from the previous experiment.

EXPERIMENTAL SET UP

14 animals performed the Morris Water Maze for 5 days; 2 did not fulfill the behavioural criteria so they were excluded from the analysis.

RESULTS

In this experiment, mice started with a similar learning curve as during the pilot experiment. However, they continued to improve strongly until day 5. This resulted in they managed to find the platform twice as fast as in the pilot experiment (Figure 9).

CONCLUSION

As hypothesized mice learnt better when the issues in the first experiment were addressed. It seems that mice in the pilot MWM experiment reach a plateau at day 3 and did not improve their performance on the following days whereas mice in the optimized version continuously decrease their average escape latency until the last day. It is possible that for mice to become really good at the task they need all variables to be close to perfect. However, they are still able to learn albeit not as well if the setup is less optimal. For long term test of memory retention, it is important that mice do in fact learn the task very well. Hence, the small details are crucial in order to get high enough power to study lasting memories.

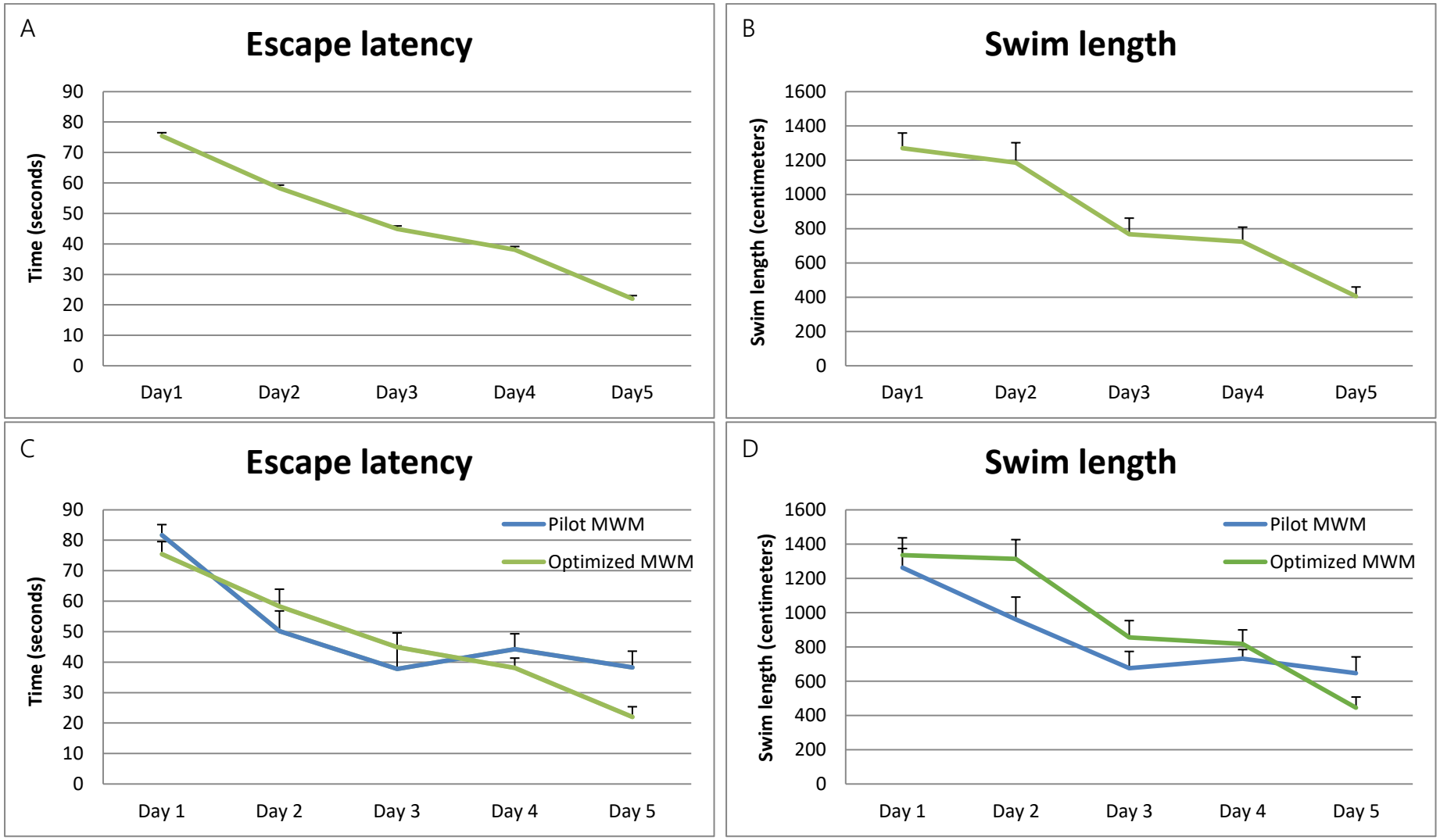

Fig. 9 A) Average escape latency and swim length (b) of animals in the optimized version of the MWM (n=12). Comparison between escape latency and swim length of animals in the pilot MWM (n=9) and in the optimized version (n=12)

4.3. WAITING PERIOD

AIM

The aim of this experiment was to investigate how stable the memory of a Morris Water Maze learning task is.

EXPERIMENTAL SET UP

In this experiment 11 female mice participated and none were excluded from the analysis. Mice performed a 5-day Morris Water Maze and later their memory was measured by a probe trial at different timepoints: days 6, 24, 38, 50 and 108 of the experiment (Figure 10).. At day 38 mice also performed, aside from the probe trial, 4 trials with the platform to measure memory in a different way

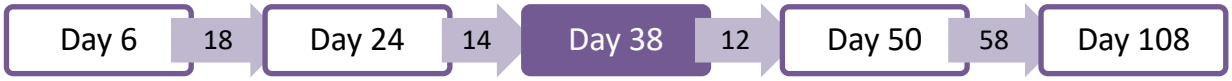

Fig. 10 Schematics of the days when a probe trial was performed. In the arrows is indicated the amount of days passed in between them.

RESULTS

The learning part of the experiment was very similar to that seen by the mice in the optimal group of the previous experiment (Figure 11).

With time, animals started to increase their distance to the place where the platform was. This increase was not highly steeped from day 6 to day 24 but it was significant if we compare day 6 to day 38, an increase of almost 20%. At day 50, after they received 4 extra trials at day 38, mice reduced their average target distance by 14%. At day 108, more than 50 days after their previous probe trial, mice also decrease their average target distance, even if this reduction was only of 11,5%. (Figure 11).

CONCLUSION

This experiment suggests that 30-40 days should be a suitable time point for assessing lasting memories. It is important not to forget on this experiment that every time the animal gets another probe trial, for example at day 24, can enhance learning or it can result in learning that the platform is not there. We hypothesise that the former reason would be the most likely.

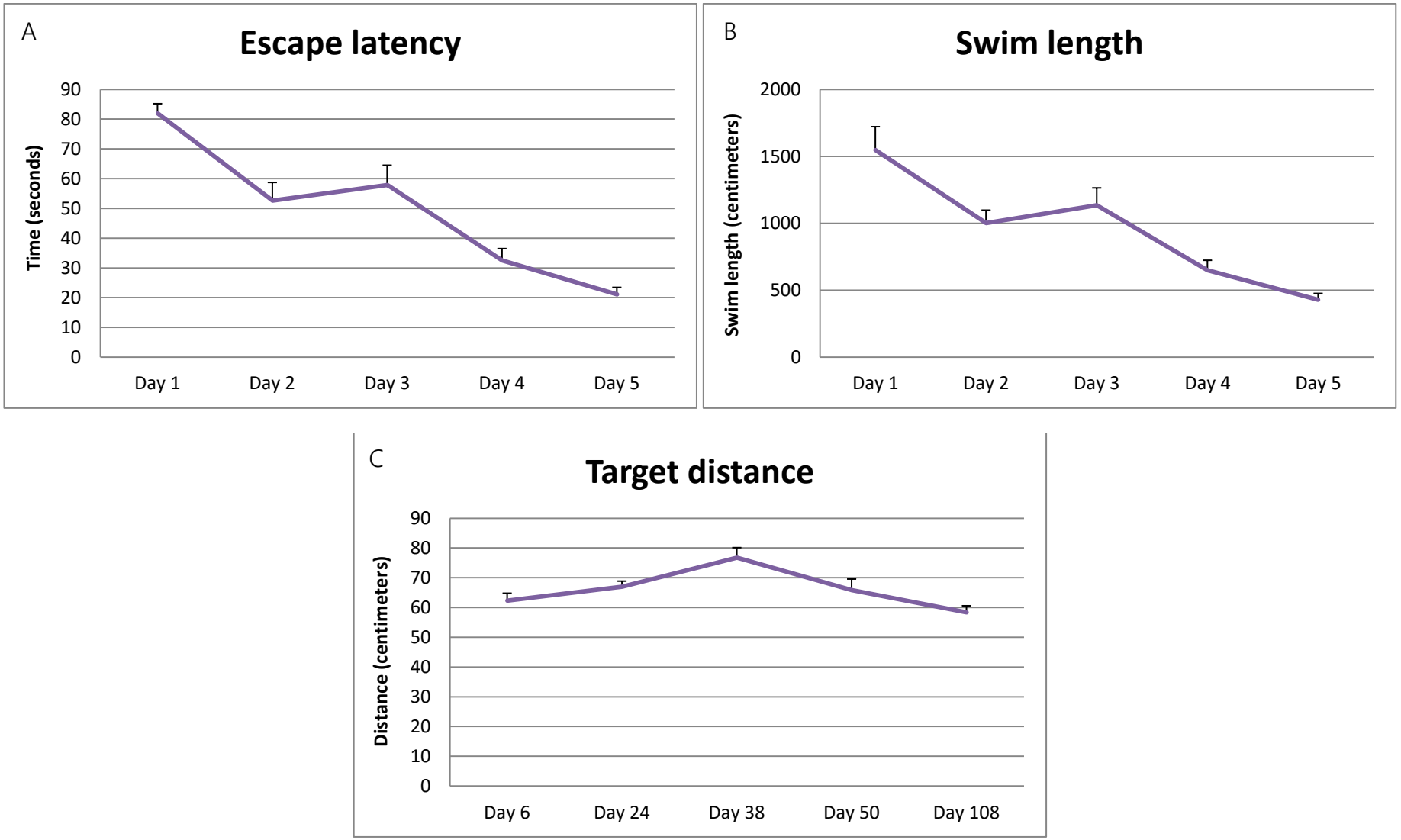

Fig. 11 A) Average escape latency (n=11) and B) Swim length (n=11) of animals during the learning phase. C) Average target distance of animals performing the probe trial at days 6, 24, 38, 50 and 108 (n=11).

4.4. AMOUNT OF C-FOS OVER TIME

AIM

In order to investigate if the early parts of learning require more c-Fos positive cells than the late part. Hence we let animals performed the Morris Water Maze either for 1 or 5 days and were sacrificed at different timepoints following the start of the first trial - 60, 90, 120, 150 or 180 min.

EXPERIMENTAL SET UP

For this experiment 11 animals were used: 1 was excluded because it did not fulfill the learning criteria defined previously; 2 were Morris Water Maze controls. 4 animals were sacrificed at day 1 at 60, 90, 120 or 150 minutes after the beginning of the experiment and 4 were sacrificed at 60, 90, 120 or 150 minutes after the first trial at day 5 (previously plotted at section 4.1).

Morris Water Maze controls are animals that experienced the pool for the same amount of time as the experimental groups. However, for these mice there was an object signaling the location of the platform. These mice were used to give an estimate of the number of c-Fos positive neurons activated by the experience of being in a different and quite stressed environment such as the Morris Water Maze. In this way, these animals might served as a more reliable baseline for the number of c-Fos positive cells.

RESULTS

Figure 12A represents the number of c-Fos positive cells in the dentate gyrus of a Morris Water Maze control mouse. Figure 12B and Figure 12C display representative pictures of dentate gyrus sections of the different timepoints analysed at day 1 and day 5, respectively.

Quantifications were made regarding the amount of c-Fos positive cells in the dentate gyrus and the CA1-CA3 region. In the dentate gyrus more cells were positive at day 1 than at day 5. Combining the day 1 and day 5 groups it seems like that neurons where most reliably activated at 90 min after exposure. For the CA1-CA3 groups the number of cells were rather stable for most groups except for 60 minutes at day 5 (Figure 13).

A

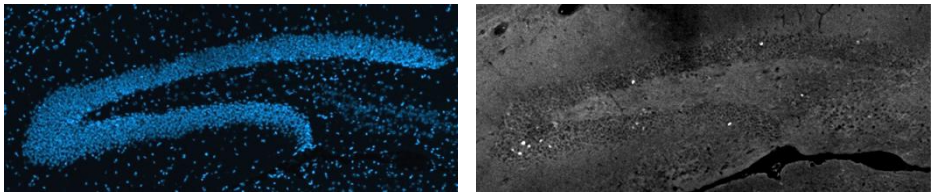

Figure 12A. Amount of c-Fos positive cells in the dentate gyrus of a Morris Water Maze control mouse. DAPI, in blue, is labeling the nucleus of the cells and c-Fos, in black is marking the c-Fos positive cells.

B

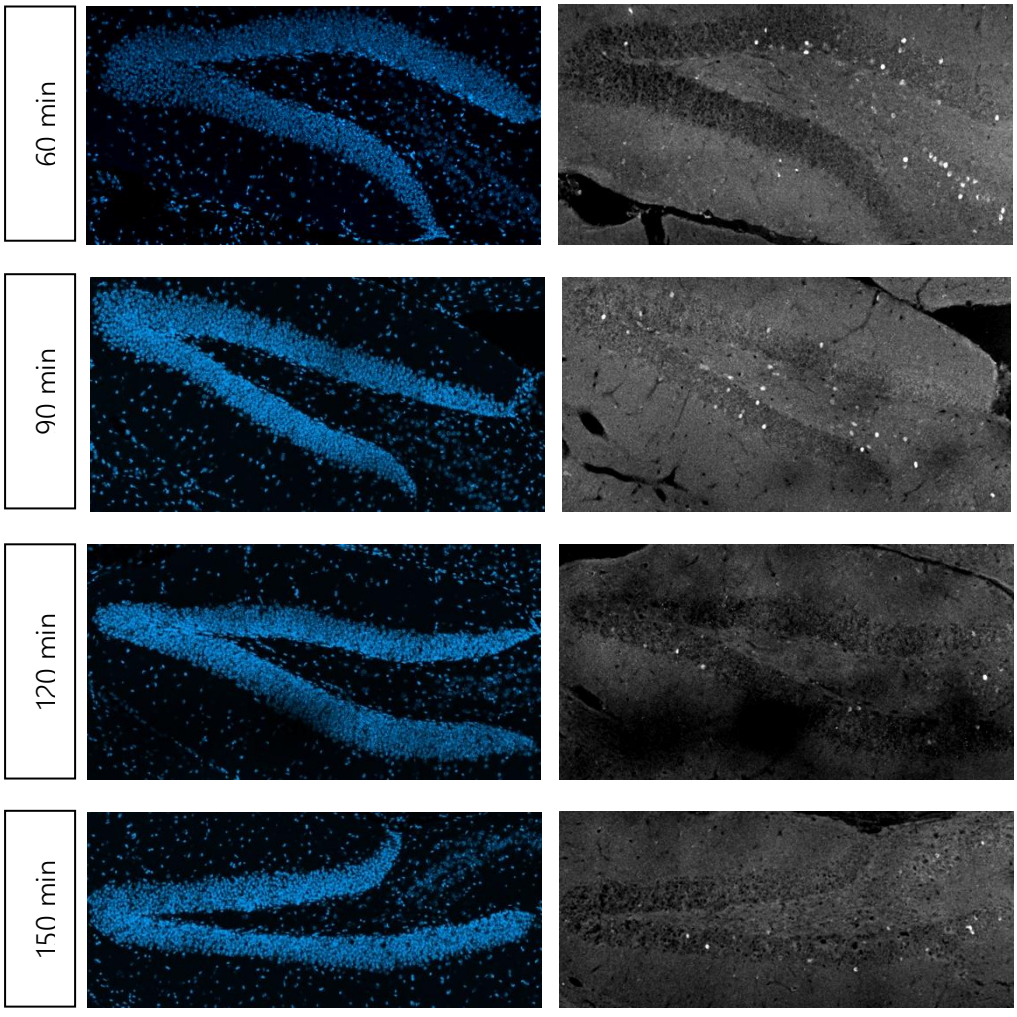

Figure 12B. Amount of c-Fos positive cells in the dentate gyrus of animals sacrificed at different timepoints (60, 90, 120, 150 minutes) after the beginning of the experiment. DAPI, in blue, is labeling the nucleus of the cells and c-Fos, in black is marking the c-Fos positive cells.

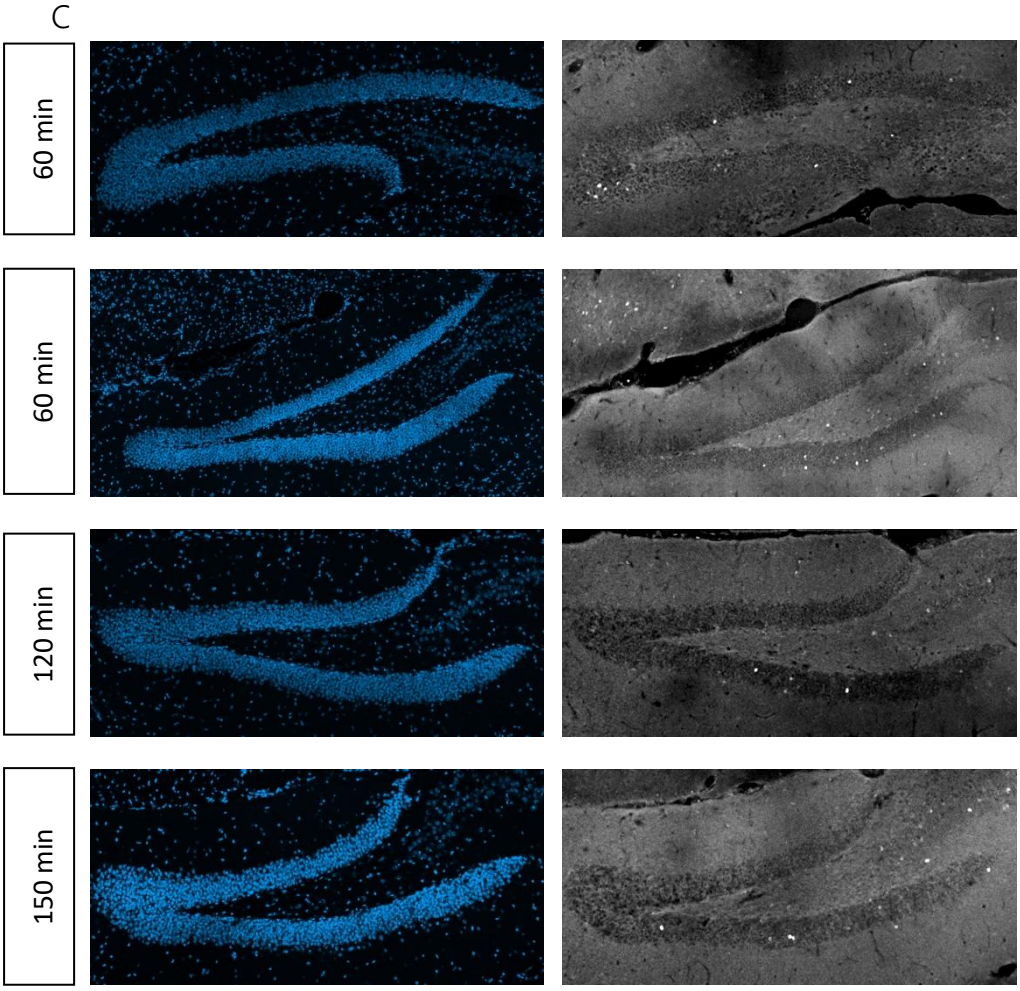

Figure 12C. Amount of c-Fos positive cells in the dentate gyrus of animals sacrificed at different timepoints (60, 90, 120, 150 minutes) after the first trial at day 5. DAPI, in blue, is labeling the nucleus of the cells and c-Fos, in black is marking the c-Fos positive cells.

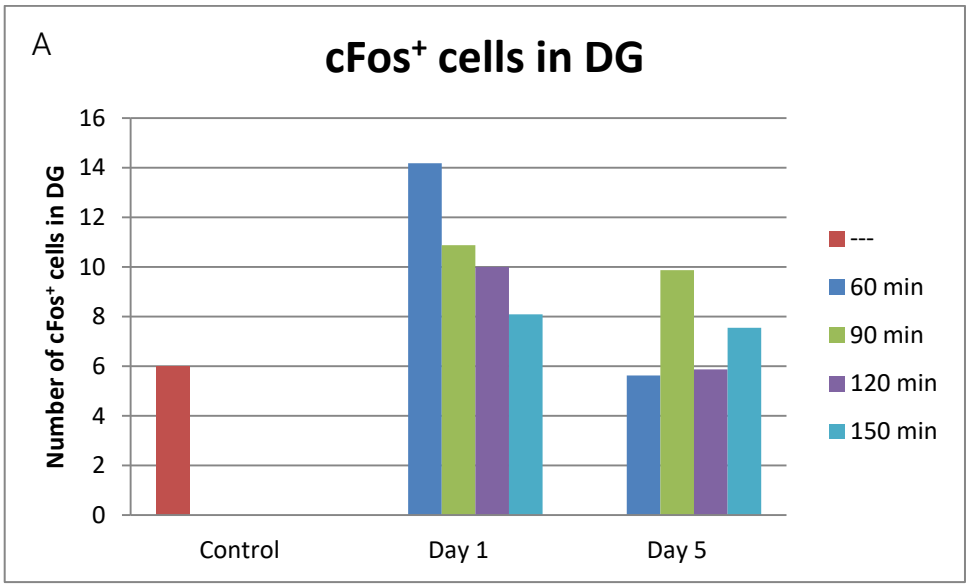

Fig. 13 Quantification of c-Fos immunoreactivity in the hippocampus A) Number of c-Fos positive cells in the dentate gyrus in MWM control mice and mice sacrificed either day 1 or day 5 at different timepoints

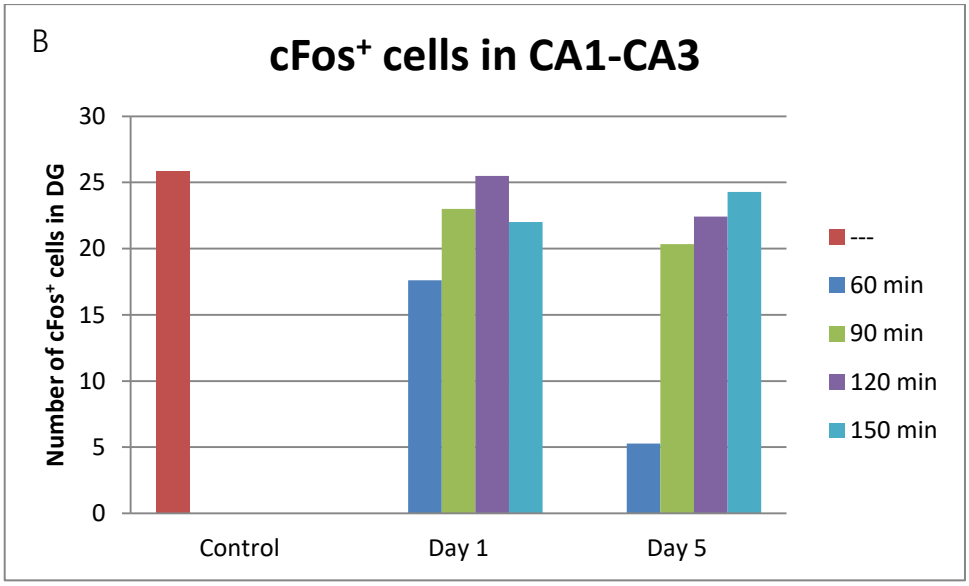

Fig. 13 Quantification of c-Fos immunoreactivity in the hippocampus B) Number of c-Fos positive cells in CA1, CA2 and CA3 region in MWM control mice and mice sacrificed either day 1 or day 5 at different timepoints

CONCLUSION

The general higher amount of c-Fos positive cells observed in the dentate gyrus at the beginning of the test might be because animals have to encounter a really new experience by performing a specific task in a completely different environment. This novelty might be enough to drive c-Fos in a fair amount of neurons such that the population of "background" cells is bigger. At day 5, fewer cells are c-Fos positive suggesting that fewer neurons are activated. This might be due to a natural selection of the neurons needed for this behavioural test.

Further, at the last day of the experiment - day 5 - there is an increased probability of having more c-Fos positive cells in the dentate gyrus 90 min after the first trial. On the other hand, it appears that the c-Fos peak in the dentate gyrus occurs earlier at day 1, which is concordant with the previous observation.

In terms of the CA1-CA3 data, the number of c-Fos positive cells in this region is quite stable over time, except when looking after 60 min at day 5. At this timepoint it can be seen a reduced amount of c-Fos positive cells which is the reflection of very little activity. Yet, shortly after, there is a strong peak. Again, this suggests that at the last day of the experiment, c-Fos might be induced by the activity of specific neurons important to the task in study and not randomly cells such it appears to happen at the beginning of the test.

The existence of c-Fos positive cells in the control animals revealed that non all these cells are specific for the Morris Water Maze. Nevertheless, this small pilot suggests that around 50% of the cells labeled in the dentate gyrus should relate directly to the memory.

It has to be noted that each timepoint is only represented by a single mouse and therefore, fairly large individual differences are to be expected.

4.5. DRUG OPTIMIZATION

AIM

This Morris Water Maze intended to optimize the drug administration to result in strong and specific labeling of engram neurons.

EXPERIMENTAL SET UP

16 mice were used in this experiment: 2 animals were homecage controls and 2 animals did not fulfill the behavioural criteria so they were excluded from the analysis. Animals were given either a tamoxifen or a 4-hydroxytamoxifen injection at different timepoints: 24h before the first trial for those receiving tamoxifen; 0-110 min timepoints for those receiving the 4-hydroxytamoxifen injection. In both cases day 2 and day 5 were the experimental days being evaluated.

RESULTS

The amount of tdTomato labeled neurons after tamoxifen injection is strikingly higher than the number of c-Fos positive neurons in the previous experiment. This strongly suggests that the majority of them would not be memory specific. The number of neurons in the 4-OHT group was much closer to the number of c-Fos positive cells. (Figure 14).

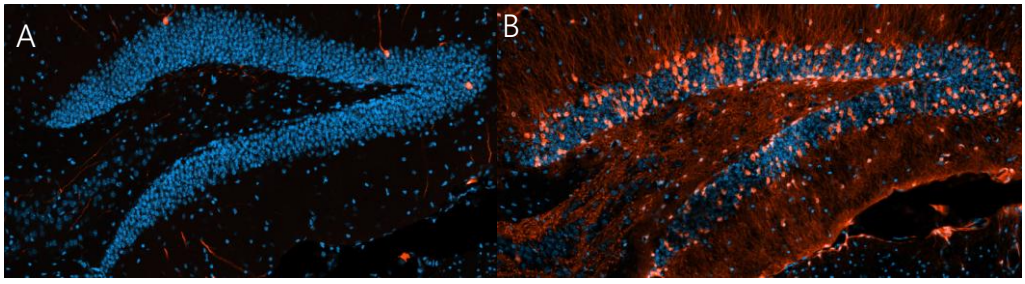

Fig. 14 Confocal microscope picture of a transgenic mouse dentate gyrus. A) Dentate gyrus of a mouse that received a 4-OHT injection and B) Dentate gyrus of a mouse that received a TM injection. DAPI, in blue, is labeling the nucleus of the cells and tdTomato, in red, is marking the cells labeled either by the 4-OHT or the TM injection.

Immunohistochemistry quantifications of thick sections (200 µm) revealed that the total number of tdTomato positive cells were higher around 60-90 min after the start of either the first trial on day 2 or day 5. It is noteworthy even in the homecage controls some tdTomato neurons were labeled (Figure 15).

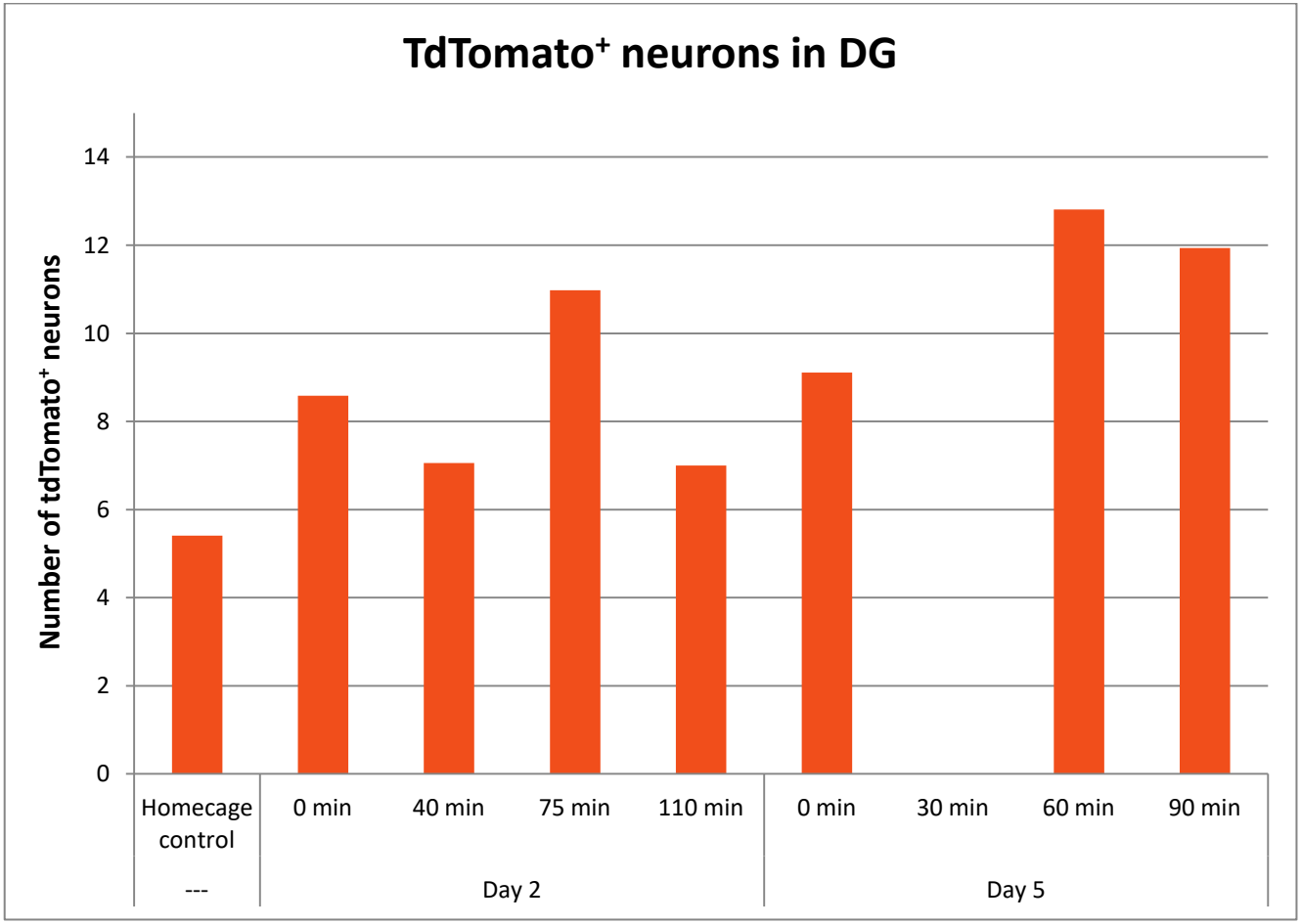

Fig. 15 Quantification on the number of tdTomato positive cells normalized for area. Animals were injected with 4-OHT either at day 2 or at day 5 of the MWM.

CONCLUSION

In regards to the form of the drug – tamoxifen or 4-hydroxytamoxifen – it is preferable to use the metabolized version of tamoxifen because the amount of labeled neurons after tamoxifen is far higher than the number suggested by the c-Fos pilot. This might be due to its long time window of action and therefore, most likely not all the labeled neurons are involved in the memory. Alternatively, 4-OHT action time window is smaller and thus the amount of labeled neurons non-specific are probably reduced. These results suggest that 4-OHT injections are more specific and therefore quantifications were only made on this form of the drug.

The existence of tdTomato positive cells in the homecage control group revealed that not all these cells are specific for the Morris Water Maze test. Nevertheless, as around 60-90 min after the first trial on day 5 the number of the tdTomato positive cells is the highest, there is an increased probability of having more memory specific cells labeled at this time point. Taking into consideration that at day 5 the task is not new for the animals, neurons activated at this day should be more specific than at day 2, where animals are still trying to understand how they can escape the maze. For these reasons, the ideal form and time to give the drug is the 4-OHT at 90 min after the start of the first trial of the last day of the learning part of the experiment.

Each timepoint is only represented by a single mouse and therefore, fairly large individual differences could be expected.

4.6. MORRIS WATER MAZE VARIATION –NORMAL AND RECALL BASED LEARNING

AIM

Combining all the results and conclusions obtained from previous work, this experiment aimed to rely on optimal conditions to analyse and study the purpose of this thesis – to study normal and recall based learning and its differences both behaviourally and molecularly.

EXPERIMENTAL SET UP

45 animals were used in this experiment. They were semi-randomly distributed to the following groups – 21 animals to the normal group, 22 animals to the recall group and 2 control mice. Out of the experimental animals, 9 and 10 were tdTomato male mice in the normal and recall group, respectively. These animals were chosen for the structural analysis and the other ones were used to give adequate power to the behavioural part of the study. The homecage control animals were both tdTomato male mice. According to the behavioural criteria, 5 animals were excluded from the normal group and 4 from the recall group ($p= 0.28$, Fishers exact test), hence there was not a significant difference in the dropout rate. Thus, behavioural data is based on 16 animals for the normal group and 18 for the recall group. Sadly, during the period between the learning phase and the delayed probe trial, 2 animals died from the normal group. For this reason, probe trial data is based on 14 animals for the normal group and 18 animals for the recall group.

Mice performed a 5-days Morris Water Maze test and 30 days later their lasting memories were evaluated with a probe trial. Mice also performed 4 additional trials at day 30, after the probe trial.

IMPORTANT NOTE

Unfortunately, when the experiment was taking place, the fire alarm of the Karolinska Institutet went off. This happen more precisely during day 4, after normal group and before recall group trials. In the animal facility the fire alarm is not audible for human beings, being instead replaced by a red light flashing constantly during a great amount of time. However, the mice could have reacted to it or to the sound of hundreds of people evacuating the building. There was also a delay in the experiment of 40 minutes.

RESULTS

From day 1 until day 3 mice were progressively learning the task since their average escape latency decrease in both groups.

At day 4, mice in the normal group that had performed the test before the fire alarm went off, also show a decrease on their average escape latency. However, mice in the recall group were trained after the alarm went off and increased the amount of time they had to find the platform. At day 5, it seems that animals in the normal group did not improve their performance, needing almost the same amount of time to find the platform than the previous day. Animals in the recall group continued to increase their average escape latency which might be a strong indicator of how strong was the negative effect of the fire alarm on them. Optimal literature and previous established learning levels were not reached for any of the groups (Figure 16).

When comparing the delayed trial at day 30 with the one at day 6 it is possible to see that mice in the normal group decrease their average target distance while animals in the recall group increase it, even if less than 5 cm of difference (Figure 17).

It seems that animals in the normal group at day 6 spent more time in the target quadrant whereas animals in the recall group have a quite even distributed time in all quadrants. At day 30 animals in the recall group spent more time in the quadrants NE and SE whereas animals in the recall groups spent more time at SW and NE. However, the difference in the recall group is not significant (Figure 18).

Interestingly there was no significant co-localization between tdTomato and c-Fos positive neurons in the dentate gyrus. Nor was there a difference depending on the treatment of the animal in the experimental groups: normal and recall based learning (Figure 19) (Figure 20). The number of tdTomato positive neurons in the dentate gyrus correlated positively with mice performance at the learning part of the experiment (R^2 = 0,4921), i.e., mice that took less time to find the platform presented more tdTomato positive neurons. (Figure 21). There is no correlation between c-Fos positive neurons in the dentate gyrus and mouse performance on the training trials at day 30 (Figure 22).

Nevertheless there was a much higher degree of co-localization of tdTomato neurons and c-Fos positive cells in the pre-frontal cortex (Figure 23) (Figure 24) which might indicate that a strong driver of the swimming performance is the neuronal assembly in this brain region. The number of c-Fos positive cells was not quantified due to the huge amount of cells and the uncertainty of the boundaries of this area.

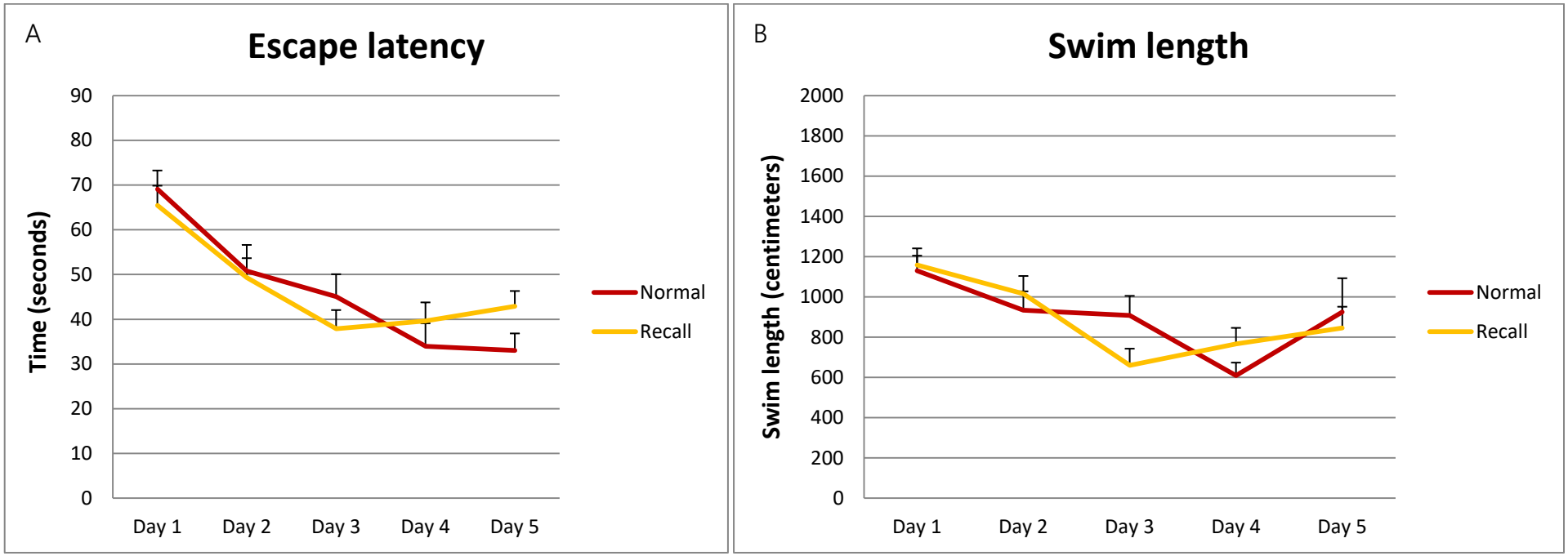

Fig. 16 A) Escape latency and B) swim length of animals submitted to normal and recall based learning Morris Water Maze test.

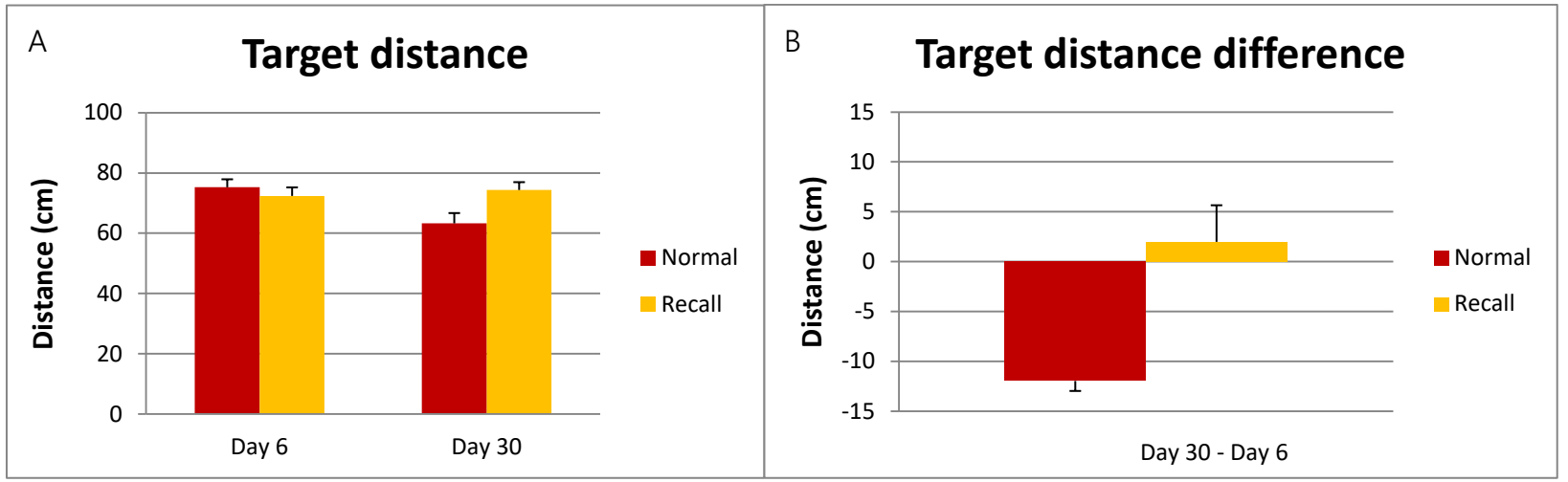

Fig. 17 A) Average target distance and B) difference of average target distance between day 30 and day 6 in both normal and recall groups.

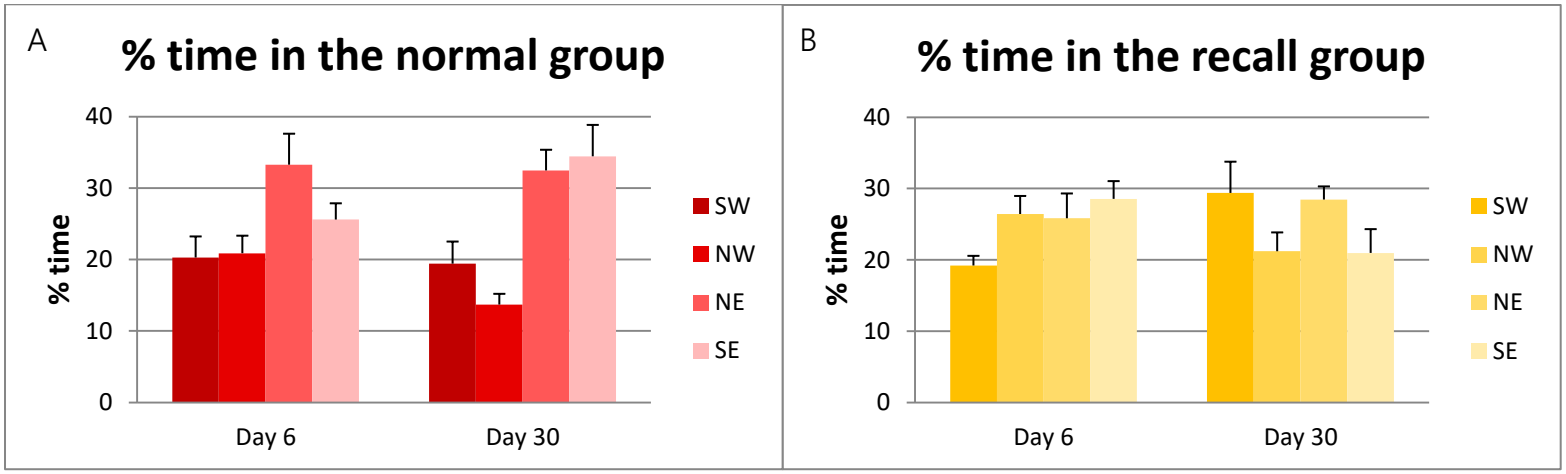

Fig. 18 A) Percentage of time in each quadrant of normal group and B) recall group at day 6 and day 30.

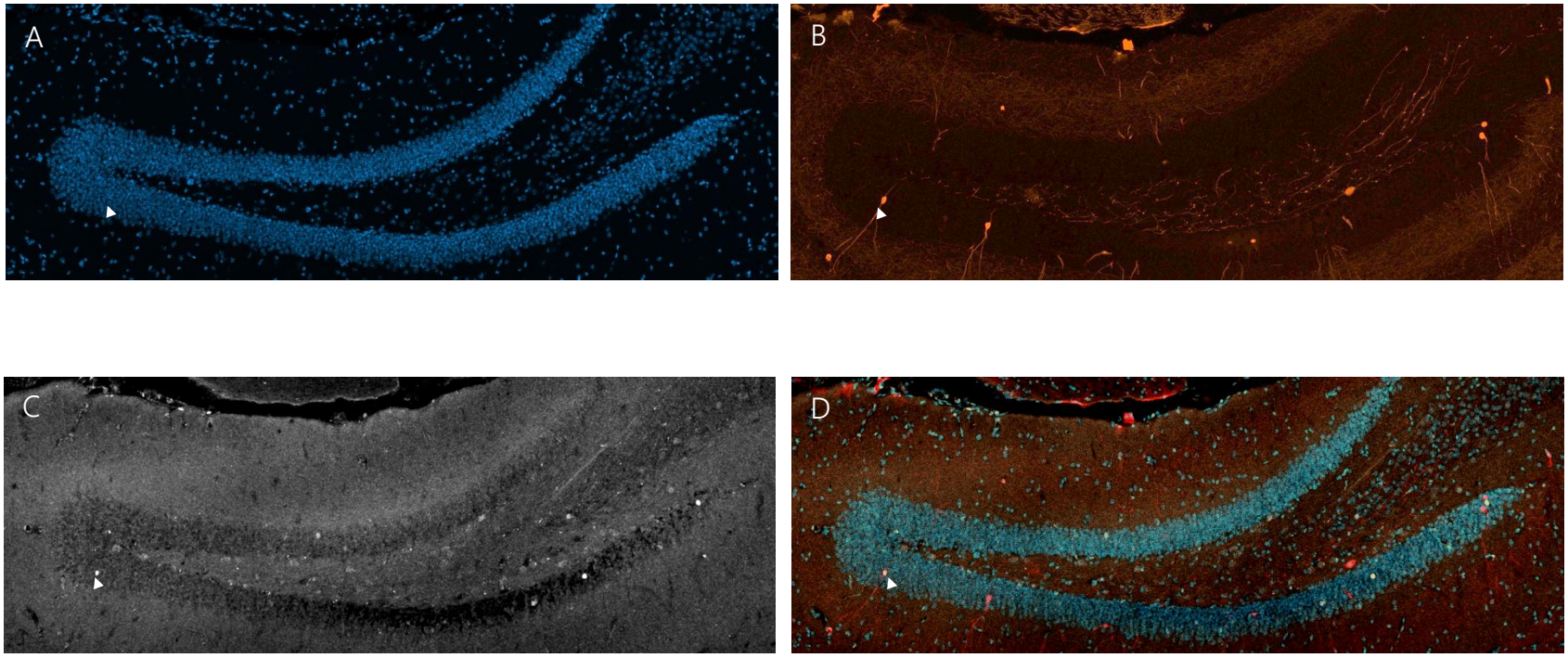

Fig. 19 Representative confocal microscope picture (20x) of the dentate gyrus of a mouse submitted to recall based learning. A) DAPI in blue staining the nucleus; B) tdTomato in red, staining the active neurons in the learning part of the experiment; c) c-Fos, in gray scale, staining the "memory" neurons active in the delayed probe trial part of the experiment; D) merge channel. White arrows indicate places where co-localization occurs.

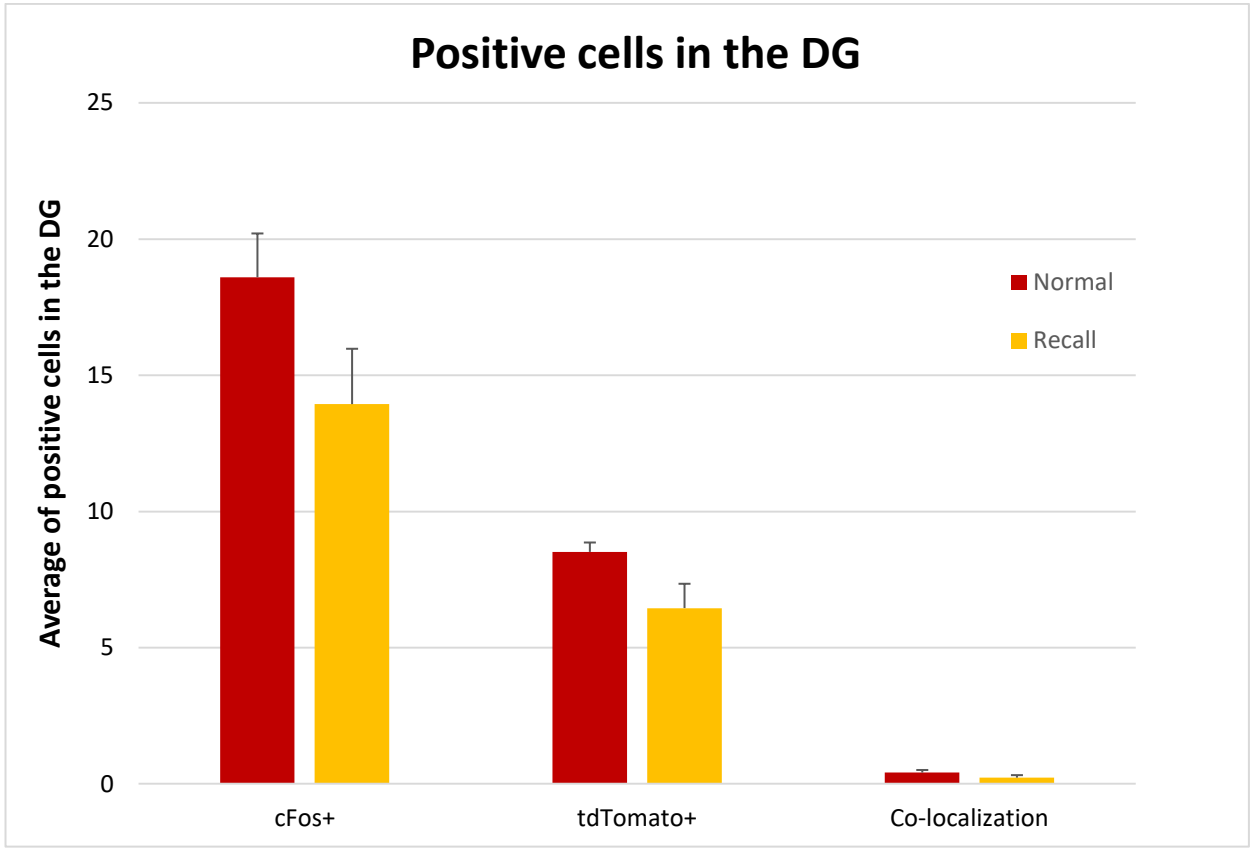

Fig. 20. Number of positive cells in the dentate gyrus for c-Fos, tdTomato and co-localizing cells, i.e, cells both c-Fos and tomato positive.

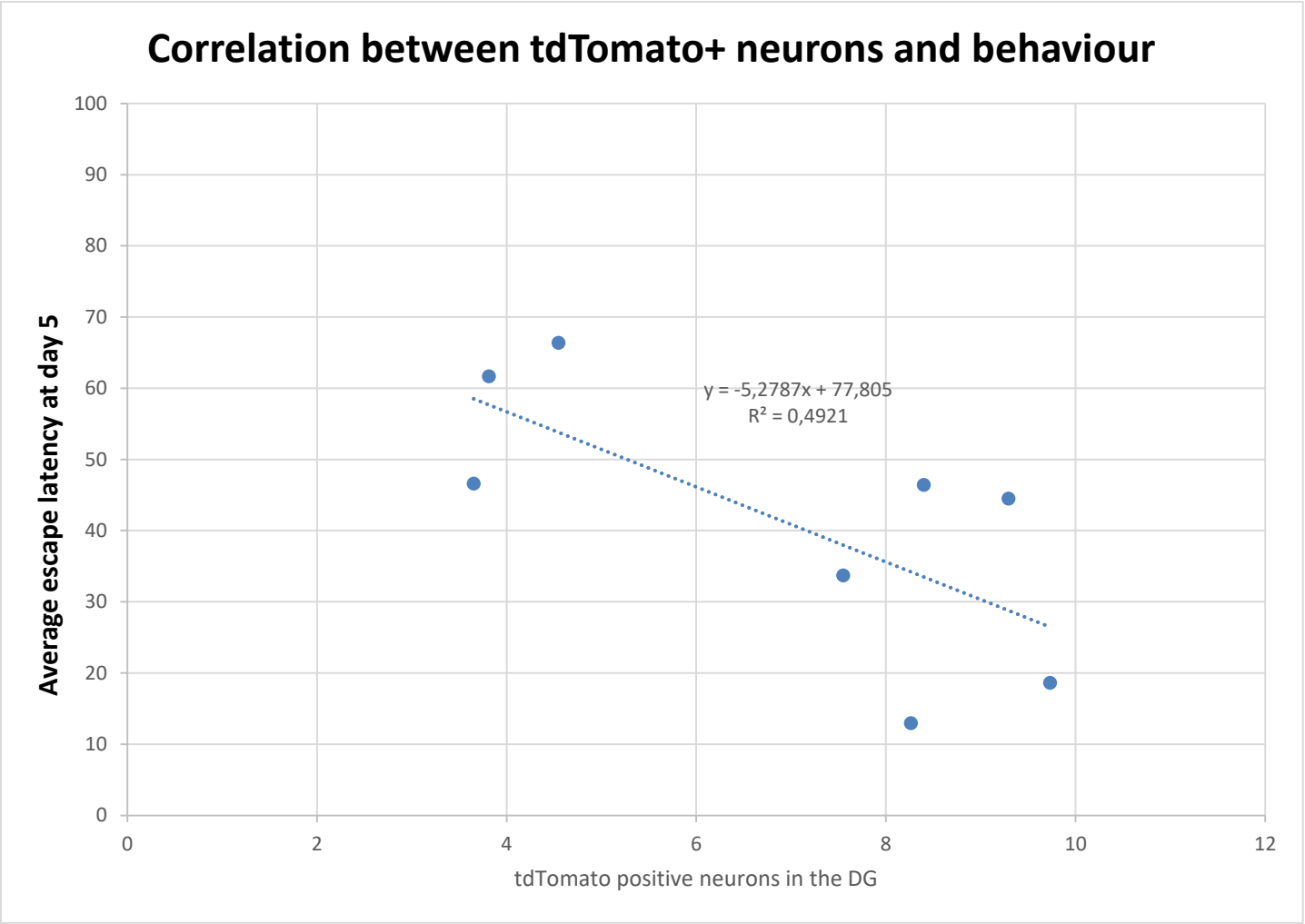

Fig. 21. Mice performance in the Morris Water Maze at day 5 as a function of the number of tdTomato positive neurons in the dentate gyrus. Number of tdTomato positive neurons positively correlates with mice performance on the Morris Water Maze at day5.

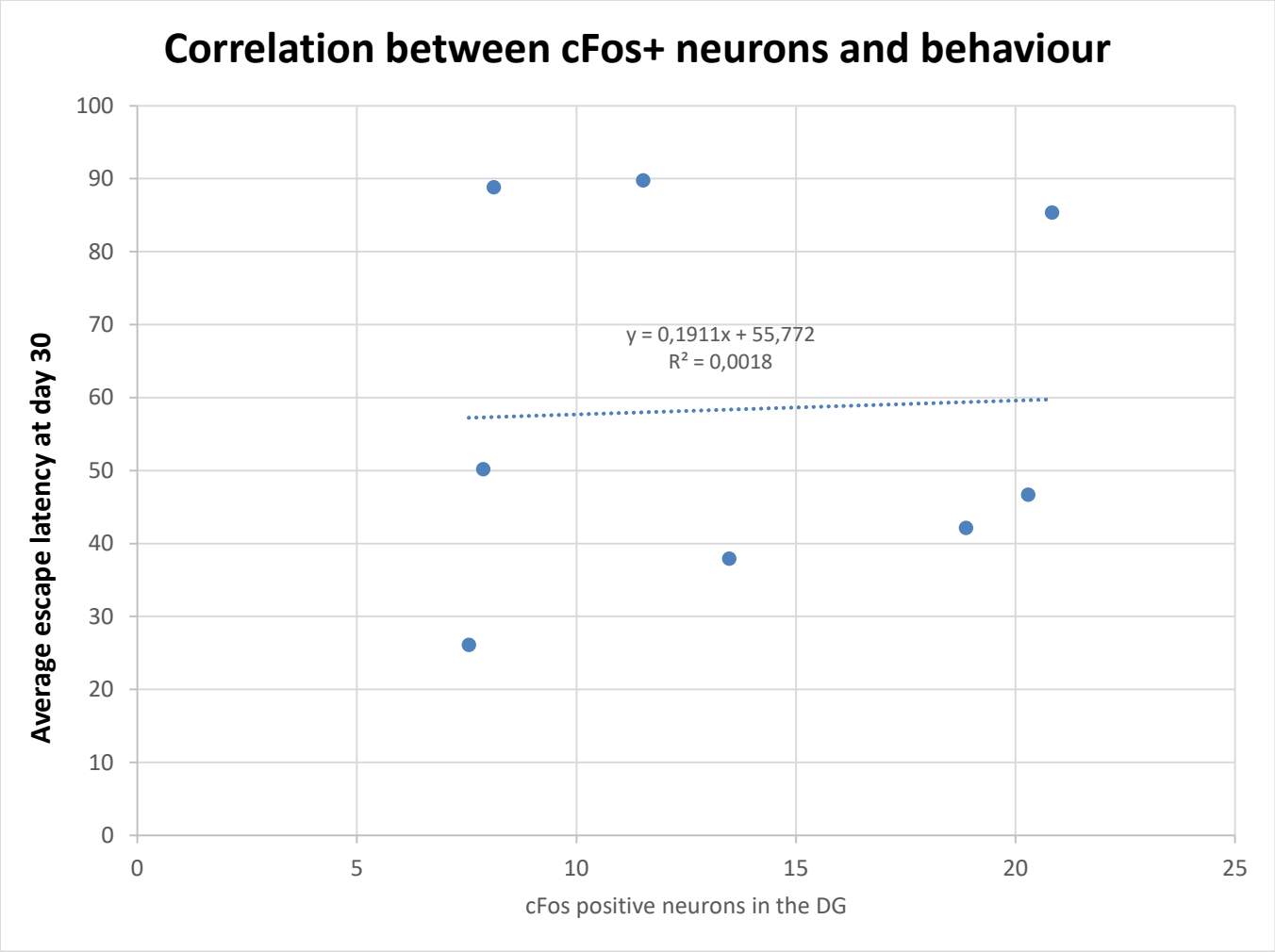

Fig. 22. Mice performance at day 30 as a function of the number of c-Fos positive neurons in the dentate gyrus. Number of c-Fos positive neurons in the dentate gyrus does not predict performance in the Morris Water Maze at day 30.

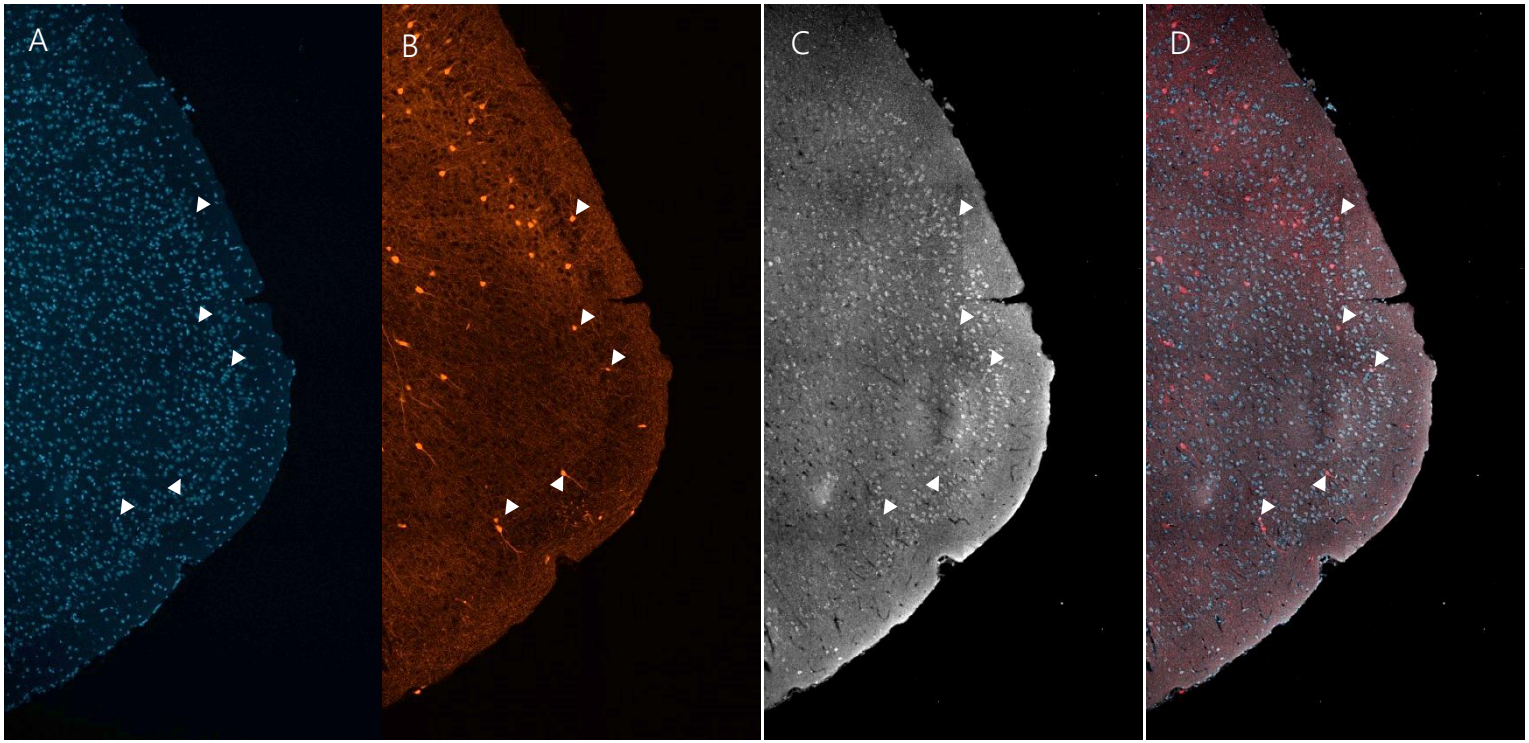

Fig. 23 Representative confocal microscope picture (20x) of the pre-frontal cortex of a mouse submitted to recall based learning. A) DAPI in blue staining the nucleus; B) tdTomato in red, staining the active neurons in the learning part of the experiment; c) c-Fos, in gray scale staining the "memory" neurons active in the delayed probe trial part of the experiment; d) merged channels. White arrows point out for example of co-localizing cells

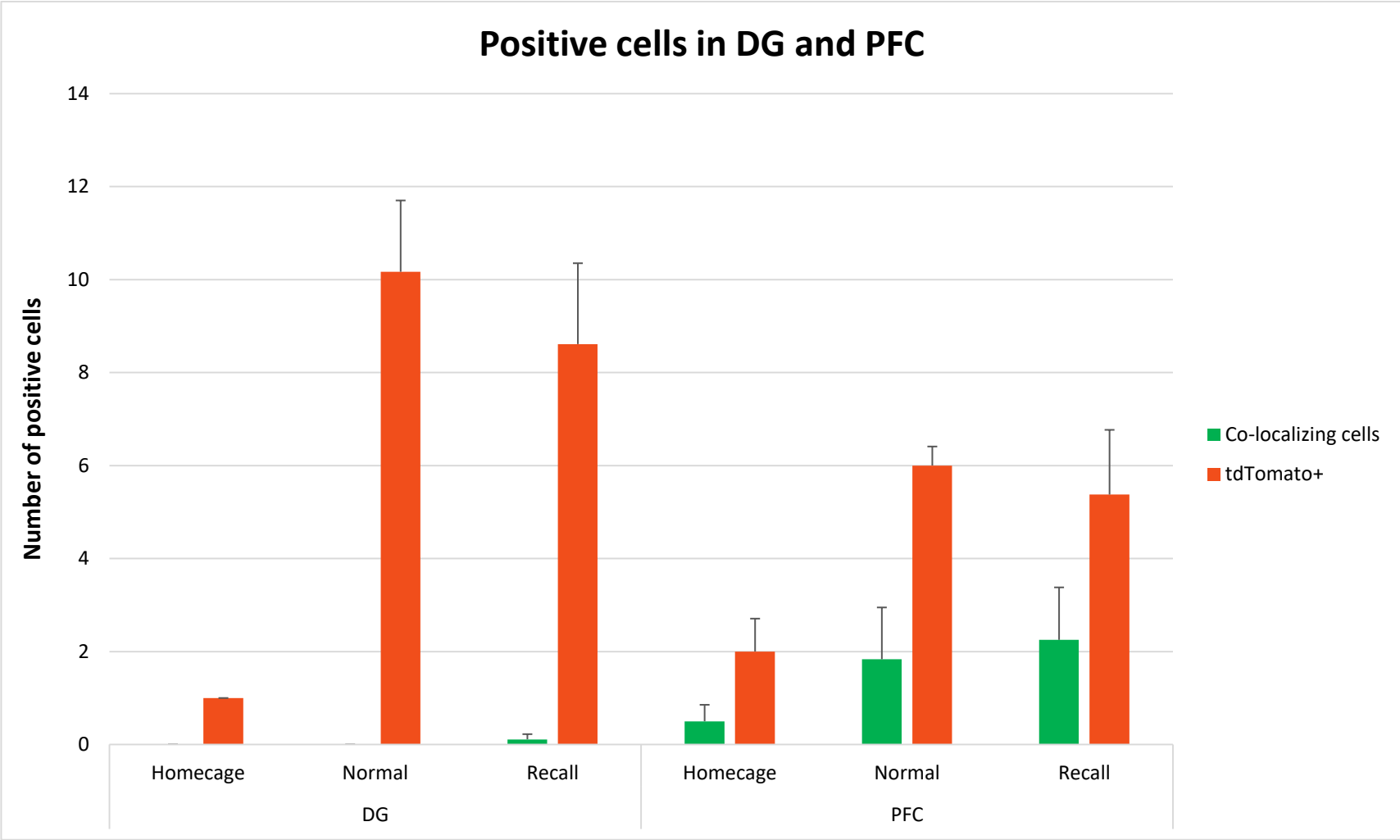

Figure 24. Number of tdTomato positive cells and co-localizing cells in the dentate gyrus and in the pre-frontal cortex of homecage control mice and mice performing the Morris Water Maze task either submitted to normal or recall based learning.

CONCLUSION

After analysing the behavioural data of the learning part of the experiment it is highly likely that the fire alarm affected the learning outcomes of the animals. Animals in the normal group did not significantly decrease their latency between day 4 and day 5 and so from the day of the fire alarm to the next day. The recall group that had the fire alarm before their day 4 swim did not improve on day 4 nor on day 5. Looking at the probe trial data, both groups showed significantly lower preference for the target quadrant than during optimal conditions previously seen.

At day 30 the normal group decreased their average target distance which is an indicator of their performance improvement. This result suggests that animals in this group had stronger learning than what was expressed at day 6, possibly due to the high stress levels still remaining. The recall group had stable but weak learning over the course of the experiment.

There is little or no co-localization of tdTomato neurons and c-Fos positive cells in the dentate gyrus. This is interesting as it indicates that when the mice are exposed to the pool after a prolonged delay period they utilize a new set of neurons in the dentate gyrus than what they did initially. However, there was co-localization in the pre-frontal cortex. This supports the notion that the stable long term storage of the memory resides at least to some extent in this brain area.

When looking at the number of neurons that recombined, around 50% of the variation in swim time at day 5, addressed by the average escape latency data, can be explained by the correlation proposed. On the other hand, there was no correlation between the amount of c-Fos positive cells in the dentate gyrus and the mice performance at day 30. Nevertheless, a considerable important region to analyse is the pre-frontal cortex and with the proposed method it is not possible due to the lack of well defined boundaries of this structure.

It is important to note that for the structural analysis the number of animals is smaller than for the behaviour because not all animals used in the experiment were c-Fos/tdTomato. Also, only animals that met the learning criterion were considered in the behavioural analysis so there are less animals than the ones set at the beginning too.

Sadly the disturbance of the fire alarm makes this test difficult to draw more conclusions. Nevertheless, this experiment looks promising so it is currently being repeated.

5. STRENGTHS AND LIMITATIONS

5.1. STRENGTHS

This project is one the first studies to address normal and recall based learning in mice and to evaluate not only the behaviour but also the engrams that are formed in response to this type of learning. Due to this, the major part of this thesis was dedicated to carefully learn and optimized the behavioural paradigm proposed, a variation of the Morris Water Maze.

Taking into consideration that there will always exist differences between mice, all external factors were controlled as carefully as possible to be similar within and across experiments. Furthermore, all animals were semi-randomly assigned to the different groups based on their genotype, sex, age and weight before the test beggin. In order to combat individual differences, in the final experiment, a relatively large sample size was used. In this way, statistical power can be achieved and likelihood of false positive and false negative results are decreased. Overall, all these factors resulted in stability of behaviour over the experiments.

In terms of analysis, tdTomato expression in transgenic mice is strong and unmistakable, facilitating the process of counting cells. In addition, antibody optimizations guaranteed a good signal to noise ratio. Furthermore, all measures were carried out by the same blind observer, thus eliminating inter-personal variation.

5.2. LIMITATIONS

There are inevitable differences between animals and it is not possible to control, in all extent, the potential external factors that also add variation to the test. Moreover, not all animals understand the task and therefore need to be excluded depending on a learning criterion, not the same in all behavioural studies of the Morris Water Maze done by far and which are described in the literature.

Regarding the structural analysis, although tdTomato expression is strong, its active time window at the time of the 4-OHT injection is still too long for specifically label only the active neurons in the learning part of the experiment. Furthermore, the brain is never completely quite and hence some neurons will be active and therefore labeled. Moreover, one of the most important brain regions to study is the pre-frontal cortex. However, this area is not really well outlined which makes it difficult to precise whether a positive cell should be classified within or not this structure.

6. CONCLUDING REMARKS

Being one of the first studies to address normal and recall based learning in mice, most of the time of this project was dedicated to the optimization of the behavioural paradigm and to understand the ideal way of addressing learning and memory neurons.

With this thesis I realize the importance of pilot experiments and the need to optimize all the possible parameters involved, especially when assessing behaviour which is, by itself, full of variability.

The Morris Water Maze set up was optimized and 90 minutes after the first trial at the last day of the learning part of the experiment was found to be the best timepoint for 4-OHT administration in order to achieve the highest amount of both c-Fos and tdTomato positive neurons and, consequently, the highest probability of specifically labeling the memory neurons involved in this task. It was also found that the memory of a Morris Water Maze task fades significantly by day 30-40 after the beginning of the experiment. Hence, this is the ideal time to investigate both behaviour and engrams in order to find improvements in lasting memory formation and the neuronal ensembles involved, respectively.

Regarding the structural analysis, several antibody optimizations and different immunohistochemistry techniques such as light-sheet microscopy or expansion protocols were also performed, regardless of not being presented in this thesis due to the lack of significance for this initial part of the project.

Sadly, the final experiment which aimed to combine the concluding results from all the pilot experiments was compromised by an unexpected fire alarm which stressed the animals and had a strong negative effect on their performance. Nevertheless, results point out that memory engrams are not the same in regards in the learning and the remembering phase. Of the regions investigated, the hippocampus correlated with the initial learning while the pre-frontal cortex could explain more of the long term memory. In fact, tdTomato positive cells in the dentate gyrus correlated with mice performance on the learning part of the experiment and co-localizing cells were seen in the pre-frontal cortex when assessing lasting memories.

Overall, I considered my thesis project really interesting but also highly ambitious. I believe this project will further give insights on the advantages of recall based learning and the underlying engrams involved and possible change the educational paradigm that we have been based on for decades. Furthermore, by learning the same task differently well, it will be possible to dissect a strong memory from a weak memory without having confounding factors such as a stronger electric shock, for example, as it happens in fear-conditioning tests. In conclusion, there are still a lot of experiments to do and most likely problems to solve in the following up of this project but that is what makes research so exciting and challenging.

7. FUTURE STUDIES

A Morris Water Maze is going to be set up with 50 mice: 25 for the normal group; 25 for the recall one. Animals from both genders are going to be used.

The size of the platform is going to be reduced from 15 x 15 cm to 10 x 10 cm to avoid any possibility of mice solving the task with procedural memory. For this reason and in order to have more timepoints to assess learning, the Morris Water Maze is going to take 7 instead of 5 days. As in the previous experiments, 24 h after the last learning trial mice will be submitted to a probe trial.

Moreover, a probe trial on the beginning of the experiment will allow a baseline for comparisons between animals initial behaviour when assessing the pool for the first time and the 24 h probe trial after the last day of training.

Animals are going to be semi-randomly divided into the two experimental groups - normal and recall. They are going to be distributed taking into consideration the following parameters - genotype, gender, age and weight -, in this order. Both genders and both groups are going to perform the Morris Water Maze in the morning and in the afternoon.

In order to guarantee that mice do not develop memory extinction or defeated behaviours the recall group will alternate recall based learning trials with days with normal trials only (Table 4).

Animals lasting memories are going to be analysed more than 30 days later after a probe trial test.

	Normal group		Recall group	
	With the platform	Without the platform	With the platform	Without the platform
Day 1	4	1	4	1
Day 2	4	0	3	1
Day 3	4	0	3	1
Day 4	4	0	4	0
Day 5	4	0	3	1
Day 6	4	0	3	1
Day 7	4	0	4	0
Day 8	0	1	0	1

Table 4. Number of trials with and without the platform per day for both experimental groups: normal and recall. Animals in the normal group are submitted to a probe trial at the first encounter with the water and 24h after the last trial of the learning part of the experiment. Animals in the recall group are submitted to probe trials every day except for days 4 and day 7.

ACKNOWLEDGEMENTS

I wouldn't have done my thesis without the support of each one of these people. Therefore I would like to leave a personal thank to:

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