IN THE PURSUIT OF THE FEAR ENGRAM: Identification of neuronal circuits underlying the treatment of anxiety disorder

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In the midst of his laughter and glee,

He had softly and suddenly vanished away—

For the Snark was a Boojum, you see.

-Lewis Caroll

The Hunting of the Snark (1876) by Lewis Carroll

Cover of the first edition by Henry Holiday.

Acknowledgements

Science may set limits to knowledge,
but should not set limits to imagination.

-Bertrand Russell

Gibran Khalil Gibran once argued that a pundit spends his life in the pursuit of knowledge, but if one day he said I have had it all, then this would be the moment of his utmost ignorance. In life, in this very journey, we all share the same start of nascence and the inevitable destination of death, yet the path one takes to complete this voyage is essentially different from one another. Many factors could influence shaping these routes, and serendipity was central in moulding mine! It all started when I have first heard the story of Phineas Gage - the most famous brain-injury survivor - at the master's defense of my eldest neurosurgeon brother. It was the first time for the regular pharmacist I used to be to start realizing that the brain is still a black box for us. It has lots of arcane details that we do not yet understand. This *per se* piqued my interest to the most and at that moment I decided to delve deeper seeking answers for the many questions started piling up. It was mainly the striking stories of Gage, patient H.M., and many others that incited me into quitting my job and dedicating my full time and concentration to search for answers to the many questions I had! And for that reason I would like to start appreciating them before those whom I have met in person during my journey and owe a big deal of recognition.

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Finally, to everyone taught me something onetime one day, **THANK YOU** from the bottom of my heart.

Phineas Gage
1823-1860

Henry Molaisan
1926-2008

O.K.

Lausanne, March 2017

TO ALL OF YOU WHO HAVE INSPIRED ME AND WONDER IF I'M THINKING OF YOU. I AM!

Abstract

Fear and other anxiety disorders are extraordinarily robust and difficult to treat. Among the most effective treatments for anxiety disorders are exposure-based therapies, during which a patient is repeatedly confronted with the originally fear-eliciting stimulus in a safe environment so that the once fearful stimulus can be newly interpreted as neutral or safe. A fundamental element for successful exposure-based therapies is the reactivation/recall of the traumatic memory, which initiates a time-limited process called memory reconsolidation, during which a memory becomes susceptible to disruption. Presently, the neuronal subpopulations and molecular mechanisms underlying successful fear memory attenuation remain completely unknown, which represents a big gap in memory research. Therefore, the aim of this work is to first identify the neuronal subpopulations that are causally implicated in effective attenuation of remote fear memories. This will help to determine whether the original traumatic memory trace has been permanently modified or a new memory trace of safety has been superimposed over the original one. The second aim is to develop a tool that allows for the isolation of the neuronal subpopulations causally implicated in remote memory attenuation, in order to be able to delineate the epigenetic and transcriptional mechanisms at play within these subpopulations. This will help to identify a molecular signature of effective remote fear memory attenuation.

The results of my research suggest for the first time that there is a small population of neurons in the dentate gyrus - that was active during the recall of fear – that needs to be reactivated during extinction to attain successful remote fear attenuation. While the inactivation of such population during extinction impairs fear attenuation, its activation ameliorates behavioral extinction. Furthermore, I have successfully established a method to isolate this neuronal subpopulation from the brain, namely by fluorescence-activated cell sorting. This tool will allow follow up studies to pursue

the quest for the molecular signature of successful remote memory attenuation. Overall, these findings could help us to better understand the intricate principles of effective remote fear memory attenuation, and thus to develop new strategies that improve the treatment of anxiety disorder.

Keywords : Remote fear memory, exposure-based therapies, fear extinction, reconsolidation-updating, contextual fear conditioning, fear attenuation, dentate gyrus, Daun02 inactivation method, hM3Dq DREADD activation method, catFISH, FACS

Résumé

La peur et d'autres troubles anxieux sont extraordinairement robustes et difficiles à traiter. Parmi les traitements les plus efficaces contre les troubles anxieux, on trouve les thérapies basées sur l'exposition, au cours desquelles un patient est confronté de façon répétée au stimulus qui provoque la sensation de peur dans un environnement sûr afin que le stimulus, jadis effrayant, puisse être interprété comme neutre ou sans danger. Un élément fondamental pour assurer le succès des thérapies basées sur l'exposition est la réactivation / rappel de la mémoire traumatique. Elle initie un processus limité dans le temps, appelé reconsolidation de la mémoire, au cours duquel une mémoire devient susceptible aux perturbations. Actuellement, les sous-populations neuronales qui sous-tendent l'extinction réussie de la mémoire de la peur demeurent complètement inconnues, ce qui représente une grande lacune dans la recherche scientifique sur la mémoire. Par conséquent, l'objectif est d'identifier ces sous-populations neuronales qui sont causalement impliquées dans l'atténuation efficace des mémoires distantes de la peur afin de déterminer si la trace traumatique originale a été modifiée de manière permanente ou si une nouvelle trace de sécurité a été superposée à la trace originale.

Les résultats de ma recherche suggèrent pour la première fois qu'il y'a une petite population de neurones du gyrus denté - active pendant le rappel de la peur – qui doit être réactivée durant l'extinction pour permettre une atténuation réussie de la peur à distance. De plus, l'inactivation d'une telle population pendant l'extinction empêche l'atténuation de la peur, alors que son activation augmente l'extinction comportementale. Par ailleurs, j'ai réussi à établir une méthode pour isoler cette sous-population du cerveau par de tri de cellules activées par fluorescence (TCAF). De futures études pourront bénéficier de cette méthode dans leur quête de la signature moléculaire d'une atténuation réussie de la mémoire à distance. Finalement, ces résultats pourraient nous aider à mieux comprendre comment l'atténuation de la peur à

distance fonctionne, et ainsi développer de nouvelles stratégies améliorant le traitement du trouble anxieux.

Mots-clés : Mémoire de la peur à distance, thérapies basées sur l'exposition, extinction de la peur, reconsolidation, conditionnement à la peur par contexte, atténuation de la peur, gyrus denté, méthode d'inactivation de Daun02, méthode d'activation de hM3Dq DREADD, catFISH, TCAF

Contents

Abbreviations

Δ freezing	Delta freezing (Freezing differential)
AcH3K9	Acetylated Lysine9 Histone3
AMY	Amygdala
βgal	β-galactosidase
BSA	Bovine Serum Albumin
CA3	CA3 field (Cornu Ammonis)
CamKII	Ca^{2+} /calmodulin-dependent protein kinase II
catFISH	Cellular compartment analysis of temporal activity by fluorescence *in situ* hybridization
CFC	Contextual Fear Conditioning
CON	Control
CNO	Clozapine N-oxide
CR	Conditioned Response
CS	Conditioned Stimulus
CTCF	Corrected Total Cell Fluorescence
DCS	D-cycloserine
DG	Dentate Gyrus
DOX	Doxycycline
DREADD	Designer Receptor Exclusively Activated by Designer Drug
EGFP	Enhanced Green Fluorescence Protein
EM	Extinction Memory
EMDR	Eye Movement Desensitization Reprocessing
E-SARE	Enhanced Synaptic Activity–Responsive Element
FACS	Fluorescence Activated Cell Sorting

FSC	Forward Scatter (aka Forward Scattered Light)
GAD67	Glutamate Decarboxylase (aka Glutamic Acid Decarboxylase)
GFAP	Glial Fibrillary Acidic Protein
H1a	Homer 1-alpha
HBSS	Hank's Balanced Salt Solution
H.C.	Home Cage
HCF	Histology Core Facility
HDAC	Histone Deacetylase
HDACi	Histone Deacetylase Inhibitor
HPC	Hippocampus
IEG	Immediate Early Gene
IHC	Immunohistochemistry
ITI	Intertrial Interval
mPFC	Medial Prefrontal Cortex
NMDA	N-Methyl D-aspartate
OF	Open Field
PBS	Phosphate Buffered Saline
PFA	Paraformaldehyde
PI	Propidium Iodide
PTSD	Posttraumatic Stress Disorder
ROI	Region Of Interest
RNA	Ribonucleic Acid
RQN	RNA Quality Number
RT	Room Temperature
SAC	Sacrifice
SR	Spontaneous Recovery
SSC	Side Scatter (aka Side Scattered Light)
SSRI	Selective Serotonin Reuptake Inhibitor
TCAF	Tri de Cellules Activées par Fluorescence
tdT	tdTomato

Tg	Transgenic
TRAP	Targeted Recombination in Active Populations
TRE	Tetracycline Responsive Element
tTA	Tetracycline Transactivator
UR	Unconditioned Response
US	Unconditioned Stimulus
VEH	Vehicle
WT	Wild-Type

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Chapter One

1 Literature Review

Paying homage to the forefathers.

In a letter to his English compatriot natural philospher Robert Hooke (1635-1703), Sir Isaac Newton (1643-1727) wrote *"if I have seen further, it is by standing on ye shoulders of giants"* [1]. Seeking collaboration through this everlasting quote, Sir Newton rephrased the words of Bernard of Chartres when he used to say that we [the Moderns] are like dwarves resting on the shoulders of our predecessors, and the fact that we managed to see farther than they did does not mean that we are better, rather it is because we have been elevated aloft on their shoulders [2]. Likewise, in this section, I will pay tribute to those who came before us to put the main pillars in this field allowing me and many others to come afterwards and pick up from where they have left off. In this chapter, I will broadly introduce some general concepts in the field of memory research. This will pave the way towards the understanding of the main research question that I have been trying to address throughout my doctoral study. Besides, in the appendix, I annex a recently published review article where I have reviewed the current studies of the field pertaining to remote memories and how they persist over time.

1.1 Memory

The diary that we all carry about with us.

-Oscar Wilde

1.1.1 What is it?

In his sorrowful poem of *"The hunting of the Snark"*, Lewis Carrol (1832-1898) – the English writer - described the tireless efforts of ten sailors trying to hunt the snark – a highly dangerous imaginary animal. The story ends when the only one of these sailors managed to find the snark, and as soon as he calls for the others and they arrive, this sailor mysteriously disappears, leading to the conclusion that the snark was nothing but an imaginary beast [3]. Everytime I think of memory, Carrol's snark comes in mind. Not because of the imaginary aspect of this animal, but – mainly – because of its elusiveness. One needs to be able to describe what one's looking for, otherwise the search efforts would be deemed unnerving. For that reason, I will try – first and foremost – to delineate what a memory could be.

According to the definition of the Merriam-Webster dictionary, a memory is the process of remembering what has been learned and retained [4]. But according to memory research, a classic definition of memory is *« a lasting change in behavior resulting from previous experience »* [5]. Variations of such traditional definition, however, emerged and tried to concisely describe memory from different aspects. In terms of different events one experiences throughout life, memory would be *«* an enduring change in behavior that results from the individual's behavioral experience *»*. Yet, upon looking at memory as mere information, then it would be simply *«* the retention over time of learned information *»*. Whereas from the perspective of internal representations (as in neuronally encoded map of the world that could guide behavior), a memory would be defined as *«* the retention over time of experience-dependent internal representations, or of the capacity to reconstruct such representations *»* [5,6].

Trying to find an exact defintion of memory is quite daunting, especially in the midst of the different views memory researchers hold for it. Amongst these, Tulving – the experimental psychologist and cognitive neuroscientist - considered memory as an entity that carries multiple senses [7]. The main characters conceptualizing these senses are as follows :

1. Memory is the capacity to encode, store, consolidate, and retrieve information (i.e. a matter of processing).
2. Memory is a « hypothetical » store where information is stored physically in the brain
3. Memory is the information itself that is stored in the brain.
4. Memory is the phenomenon of retrieving information from where it is stored (i.e. act of remebering).
5. Memory is the phenomenon of being aware that one is remembering something (i.e. the conscious awareness of the remembering action).

All of these aforementioned senses of memory might be in line with the definition of memory for the uninitiated, yet some of them did not conform quite well with several erudite memory researchers. Nadel and Moscovitch for example consider memories for their abilities of being represented, meaning that they do not exist unless expressed [8,9]. While the cognitive psychologist Daniel Schacter rejects the idea that memory is the conscious awareness of remembering since there are some implict types of memories that lack such aspect (e.g. sensorimotor skills) [10]. He, also, argues against one of the most common descriptions of memory as an effect of experience that persists over time. Schacter points out that a « bendy ruler » can be molded into different shapes, thus will be able to hold these shapes over time, yet it lacks the essence of being a memory due to the lack of representation i.e. encoding the shape, storing it, and retrieving it later on. It could be indicated, however, that it carries a memory of shapes in the laypeople's metaphorical use of the term. But like that, this so-called memory is nothing but to allow users to draw these shapes, yet this bendy ruler does not possess them itself and, therefore, is unable to store and retrieve these shapes itself [10].

It seems that the more we delve deeper into finding a core concept for memory, the more we appreciate how difficult such undertaking is. However, and despite these

disparate views, most scientists widely agree on certain characteristics their versions of memory undisputedly share. Therefore, and in my attempt to seek the core of memory as a concept, I decided to characterize what a memory could be rather than just trying to find a precise definition for it. Ultimately, and according to my understanding to the field, memory is **the neurocognitive capacity to encode, store/consolidate, and retrieve information, where this stored information could, sometimes, persist overtime**. These basic neurocognitive capacities will be addressed in further details in the following subsections.

1.1.2 The Engram

By definition, an engram is the physical record of a memory, and sometimes referred to as the « memory trace » [4]. It was Plato who pioneered in proposing such understanding of memory engram. In one of his dialogues – *Theaetetus* ca. 396 B.C. – he argued that a memory is like a tablet of wax, where events can be etched upon. Yet when forgetting is taking place it is merely because such engravings had been scraped off [11,12]. Thousands of years later, Richard Semon (1859-1918) – the German zoologiat – coined in his book « *The mneme »* the word « engram » having its meaning from the greek roots for « something converted into writing »[13]. However, in its debutant as a term, Semon referred to engram as a general theory that keeps track of experience-dependant records in living organisms be it neural, developmental, and genetic memory in all types of tissue [14]. In the mneme, he suggested two laws whereby memory functions : First, the law of engraphy, where all excitations within an organism act engraphically, i.e. they leave behind a mnemic trace; second, the law of ecphory, where the mnemic trace or engram can be reawakened of its latent state manifesting the activity used to be there during engraphy [14].

It was not until half a century afterwards when the psychologist and behaviourist Karl Lashley (1890-1958) revived the term in his famous « search of the engram » [15]. Surprisingly, however, in this enormously cited paper of his there is not a single mention to Semon in it. In his search for the engram – and entire career as well – Lashley lesioned many brain regions of experimental animals in the hope of localizing

the trace. Amongst the outcomes he concluded from all the experiments he performed, Lashley summarized the findings into two principles : First, the equipotentiality principle, where he observed that all cortical areas are equal for learning and one can substitute the other to serve learning ; second, the mass action principle, where the learning deficit is roughly proportional to the amount of tissue destroyed, rather than its position, and that the effect of the lesion is proportional to the complexity of the task performed by the brain-lesioned animals [15,16]. These findings let him to conclude that *« This series of experiments…has yielded a good bit of information about what and where the memory is not… I sometimes feel, in reviewing the evidence on the localization of the memory trace, that the necessary conclusion is that learning just is not possible… Nevertheless, in spite of such evidence against it, learning does sometimes occur »* [15].

The work of Lashley and his students highlighted important basic issues that we – as memory researchers – are still trying to figure out. Amongst these issues [6]: Firstly, the existence of an engram. Secondly, the localization of the engram, which raised many hypotheses depending on the complexity of the engram and type of organism, where some engrams might be localized to a specific region, yet others could be highly distributed in cell assemblies/circuits – different population of neurons that are co-active during the encoding the memory – or some engrams could even shift their location from one region to another like what happens during the consolidation of an episodic memory to become independent of the hippocampus [17]. Thirdly, the differentiation of function, where the regions supporting the engram might be important in one phase of the memory and not the other, e.g. essential during encoding but not in storage or retrieval. Fourthly, the transformation of the engram, where the engram itself could change upon simple alteration(s) to parameters of encoding or retrieval. Finally, the level of the engram, where the anatomical location is not *per se* the main hunt, but the what happens on the molecular and cellular levels of it. This last aspect is of particular importance, especially since the core of the engram might be located on a distributed circuit level, yet synapses and cellular molecules may contribute to the upkeeping of the physical record of the experience i.e. the engram [6,18].

All the aforementoined issues around the engram, stimulated present-day scientists to consider several criteria – reviewed in [19–23] – for an engram to deserve its title.

These criteria are as follows : 1. A persistent change taking place in the brain as a result of an experience or an event ; 2. Retrieval of an engram should occur by artificially activating the neurons active during the encoding even without the external stimuli that used to be present; 3. Blocking the activity of the neurons active during the encoding would impair the memory retrieval ; 4. The engram should exist in a dormant latent state in between encoding and retrieval, without which blocking and retrieving would by definition not be possible. Addressing each of these criteria should therefore shed light on the functionality of the activated populations in the brain and their contribution to the engram.

1.1.3 Types of memory

Multiple systems and processes govern memory giving it different functional roles, and classifications. For example, memory can be categorized based on the duration of its storage system. A short-term memory can hold information that is active in mind for a limited time period, whereas a long-term memory stores information durably [24]. Another distinction memories can be classified into, is based on the consciousness of knowing the memory itself. It is simply the difference between « Knowing that » and « Knowing how ». In « Knowing that », it pertains to the conscious recollection of facts and episodes, i.e. the information can be declared, and that is called « declarative memory ». Whereas in « Knowing how », it refers to skills and procedures with the capacity of implicit learning through repeated exposure to a task or a stimulus that does not require conscious thinking of it, and this is called « non-declarative memory » [25].

The declarative/non-declarative distinction is conveniently subdivided to more specific classes according to the type of information stored. Firstly, the declarative memory can be either : ***1. Semantic***, where it is devoted to the knowledge of facts like words, objects, and concepts ; or ***2. Episodic***, where it is dedicated to storing information about events with their spatio-temporal context, i.e. information about an experience locked to a particular time and place [26]. Secondly, the non-declarative memory comprises : ***1. Priming***, where repeated exposure to a stimulus can eventually modulate/enhance information processing; or ***2. Procedural learning***, which denotes the capacity to progressively and automatically acquire skills [27].

Additionally, in amnesic patients they tend to usually have an impairment of one aspect of memory. For example, they can retain information for a short-time, i.e. they can have intact short-term memory, yet they fail to preserve the information for a durable time, i.e. they suffer from impaired long-term memory. Furthermore, these amnesic patients could also have impairment to their memories in a single aspect like failing to encode and retrieve episodic information, yet their semantic and procedural memories could be spared. The memory deficit usually correspond to the damage of the brain region that is essential to the process of a certain type of memory [28].

1.1.4 Memory Processing

Like living beings, memories do possess a timeline of their own, where acquisition is the moment of their birth, and depending on their types, they pass by some of these major phases of consolidation, retrieval, reconsolidation, persistence and extinction (discussed under the following section). All these aforementioned steps could be collectively denoted as processes the memory encounters during its « lifetime ». In this subsection, I briefly introduce each of these memory processes.

I. Acquisition

Memory acquisition is what marks the initial phase in the formation of the memory. It is the process by which new information is converted into a memory trace representing a progression of learning [6]. This process is what Semon referred to as the « engraphy », where the memory is engraved [14]. Acquisition in the literature usually refers holistically to learning, and the main subprocesses that constitute it are: firstly, encoding, which converts what is being learned from one language/code to another ; and secondly, registration, which is the recording of such conversion into an engram [29]. This second phase of acquisition plays an important role for differentiating between what information will be transitory, and what will last longer, and this in turn depends on the learning paradigm and protocol used during acquisition. The higher the number of repetitions or the aversiveness of the stimuli during acquisition, the longer the information would last [6]. Furthermore, the brain regions responsible for the acquisition are quite diverse depending on the type of

memory to be acquired. For example, declarative memories engage widely distributed areas including cortical regions, the hippocampus, and the amygdala, where it has been shown through lesion studies that some of these regions are indispensable for the acquisition process to take place [30–32].

II. Consolidation

The indispensable dialogue for memories to persist.

The process of consolidation is the phase during which the stabilization of memories takes place. It is a gradual stabilization that follows the acquisition process [33]. The Roman rhetorician Marcus Quintilianus – mostly known as Quintillian (ca. 35 – ca. 100 AD) – was the first to note that memories' strength increases with time. He reported that *"the interval of a single night will greatly increase the strength of the memory"* and hypothesized that *"... the power of recollection .. undergoes a process of ripening and maturing during the time which intervenes."* [17]. The consolidation term itself was coined from the Latin root for "to make firm" by the experimental psychologist Müller (1850-1934) and his student Alfons Pilzecker in 1900 [34]. From that moment scientists conducted an enormous amount of work to understand how memories stabilize. The outcome showed that the consolidation process is not necessarily completed within a short time following acquisition, as it may continue over weeks, months, or even longer [17]. The process starts with a stabilization of the information at the local level of neurons and their synapses, which is usually referred to as "cellular consolidation". It is a local, and time-dependent stabilization at the level of the neuronal circuits that encode this memory. It has been shown that this process lasts for several hours, during which it can be disrupted using protein synthesis inhibitors [35]. These new proteins that are synthesized are believed to be central for the long-term modifications of the local synapses and are thus essential for the first phase of stabilization [36]. This initial phase is soon followed by another one, namely, "system consolidation", where gradual recruitment and continuous reorganization of distributed brain circuits take place [37].

III. Reconsolidation

In declarative memory, it is believed that it initially engages the hippocampus during its formation and the first step of stabilization, but the system consolidation renders its storage in the long run dependent on the cortical circuits and independent of the hippocampus, where such dialogue between these brain regions is quite essential at that step [38].

Many explanations have been posited concerning the importance of consolidation and why memories are not stabilized instantaneously. One explanation speaks in favor of saving the brain's capacity from unwanted useless items, and that this time window of consolidation provides essential malleability of the memory to be easily associated with other inputs facilitating more efficient encoding and registration [39]. Furthermore, and once consolidated, a long-term memory has been shown to be "disruption-resistant", i.e. it is no longer vulnerable towards protein or RNA synthesis blockers [40]. Intriguingly, it was believed for a long time that a consolidation of a memory starts and ends only once. However, it has been observed that a memory can be reconstructed again upon recalling the stored information of the past once again [41]. Such reconstruction of the memory raised the possibility that the retrieved memory might need to be consolidated again in order to persist [42]. Such cellular consolidation that follows retrieval has been termed "re-consolidation" and was indeed experimentally shown to occur [42–44]. However, whether reconsolidation is a generalizable concept across different types of memories is still under debate and extensive investigation. For instance, it has been shown that the cellular mechanisms underlying the consolidation of a novel trace and the reactivated are essentially different [18,45].

IV. Retrieval

This process of retrieving a memory is basically the reconstruction of stored information, i.e. the recall of a stabilized memory, where this memory becomes active once more from its latent stabilized state [6]. It was proposed that an non-retrieved memory should be called inactive, whereas retrieved and short-term memory ones are indeed the active ones [46]. For retrieval to be successful, availability of appropriate cues need to be provided. The retrieval will be more efficient if these cues are essentially the ones that were present during the acquisition, i.e. when the retrieval environment resonates with the one of the acquisition [47]. Nevertheless, and using modern tagging and manipulating techniques, it has been shown that the retrieval of an inactive memory can also be achieved without providing the same cues that were present during acquisition (reviewed in [19–21]). Moreover, and concerning brain regions responsible for retrieval, retrieval is also dependent on the type of memory. For declarative memory for example, many connected areas are vital for the retrieval including the prefrontal cortex as well as the hippocampal regions [48–51]. Finally, it is noteworthy that the retrieval can be enhanced using certain nutrients like glucose [52], and can be impaired by corticosteroids or under stressful conditions [53].

V. Persistence

This part of a memory is process is discussed in detail in the review attached in the appendix. But briefly, persistence is what makes the memory endure over time. When the information is stored as "hardware alterations" in the circuit, then this would provide continual existence of the memory. Many issues are discussed about this issue, firstly, how does the memory outlast the molecular components that helped in its stabilization, i.e. the proteins that were synthesized and have a limited lifespan – or even the post-translational modifications of them – during the stabilization phase [6]? One of the proposed suggestions is a positive molecular feedback loop that once activated it regenerates the molecular change again and again resulting into a stable expression of the needed proteins [6]. This has been shown by the studies carried out to account for the immunity towards molecular turnover through use-dependent neuronal change [54–59]. Also, spine density, as well as the strength and number of synaptic connections that are formed between neurons involved in the acquisition and

afterwards during the systemic reorganisation have offered a possible explanation for memories to persist [60–63]. And despite the synaptic connections themselves are sustained by proteins, yet the molecular feedback loop mentioned earlier supports these connections. Moreover, the integration of newly-born neurons support the functional circuits to sustain the memory trace [64–66]. However, it is still yet to be addressed how the incorporation of new neurons into the circuit - and after a while the old hardware will be completely replaced – will keep hold of the old trace?[6]

1.2 Associative Learning

Forming a mental link between different events

1.2.1 Classical Conditioning

Forming « associations » was amongst the many aspects with which philosophers and brain researchers use to study the mind. It was the Greek philosopher Aristotle (384-322 BC) who proposed that similarity, contrast, and contiguity of images subserve recollection. The Russian physiologist Ivan Pavlov (1849-1936) was a pioneer in expanding on this concept when he performed his famous experiment of classical conditioning. After a series of experiments, Pavlov and his dogs – they were many – demonstrated that a subject can form an association that one stimulus predicts another. He named this type of experiments classical conditioning, where a type of training is carried out in which two stimuli, the conditioned stimulus (CS) and the unconditioned stimulus (US), are paired with each other, so that the CS comes to evoke a conditioned response (CR), which is similar to the unconditioned response (UR) elicited by the US. In Pavlov's experiments, the CS was a sound of a metronome, and the US was the food presented to the dog. So he observed after many repetitions of giving the food to the dog while the sound of the metronome was on that the dog started to salivate in response to the sound of the metronome even without giving the food afterwards. Pavlov then concluded that if a stimulus is to be presented in the dog's surroundings while it is being given the food, then that stimulus could become associated with food and cause salivation on its own. Afterwards, Pavlov paid much attention into investigating the importance of timing and order of the CS and US. He showed that the most effective style of conditioning « delay conditioning » would be if the CS is presented first and then the onset of the US should come before the offset of the CS or coincide with it [67].

1.2.2 Pavlovian Fear Conditioning

A reminder of the "little Albert" inside every one of us.

Terming it as a « passive defensive reflex », Pavlov did not keenly study fear and how it is augmented by experience, he just noted that a memory of a traumatic experience could linger for a while and can block the expression of other acquired behaviors [67]. John Watson (1878-1958) – the English behaviorist – however, had a great interest of studying fear. And while Pavlov used dogs in his experiments, Watson did his on human infants [68].

Watson and his student Rayner, had little Albert – a nine-months old infant – in an experiment to mimic what Pavlov did with his dogs but to make Albert associate fear with another stimuli [68]. They observed Albert's reaction upon exposing him to a series of different stimuli including a white rat, a rabbit, a monkey, masks and burning newspapers. Albert initially showed no fear to such items, however, and after repeatedly pairing a white rat with a loud noise of a hammer hitting a metal pipe, Albert began to cry as soon as he laid his eyes on the rat. Thus, Watson and Rayner reported that *"The instant the rat was shown, the baby began to cry. Almost instantly he turned sharply to the left, fell over on [his] left side, raised himself on all fours and began to crawl away so rapidly that he was caught with difficulty before reaching the edge of the table."* [68].

The little Albert's experiment led many scientists to adopt pavlovian fear conditioning into studying emotion and the brain regions responsible for such type of learning [6]. A tiny modification on the original experiment was applied where the rat is the one that is conditioned, and human subjects act as observers only – in all ethical likelihood for the better. There are many varieties of this Pavlovian fear conditioning where it depends on what is used as the CS. In the auditory fear conditioning the rat (or the mouse) hears a tone (the CS) that is followed immediately by an electric foot shock (the US), in this type of conditioning the tone elicits the fear response which is the freezing of the animal (the UR) even in the absence of the US [69–71]. Another type of Pavlovian fear conditioning replaces the tone with the context the animal receives the shock in, known as « contextual fear conditioning » [72]. Using these

variants of fear conditioning along with other experiments of cellular physiology, pharmacology, and brain lesioning, scientists proposed a model of fear conditioning where the amygdala as well as the hippocampus appear to play a central role.

1.2.3 Extinction

During one of his classical conditioning experiments, Pavlov observed that the CS is no longer eliciting a CR in one of the dogs. The dog was no longer salivating in response to the sound of the metronome. He afterwards discovered that upon frequently presenting the CS, i.e. the sound of the metronome for long periods to the dogs, the salivation diminished. He termed such phenomenon « experimental extinction » [67]. By definition, experimental extinction is the decline in the frequency of the intensity of a conditioned behavior following the withdrawal of reinforcement. This essentially happens as a result of the rearrangement of the relationship amongst previously associated stimuli, so that these stimuli are no longer capable of eliciting the once formed CR [6]. However, what Pavlov also observed is that the CR may recover at a certain point after extinction – termed as spontaneous recovery – without any additional steps of training. This made him propose that what happens during extinction is essentially a process of internal inhibition to the CR [67].

Such phenomenon of experimental extinction has been extensively used in psychotherapy especially in the treatment of posttraumatic stress disorder (PTSD), as well as to understand the circuits that are dedicated to the learning of the fear, i.e. its acquisition and to differentiate it from the circuits that are responsible for the extinction, i.e. the relearning or new learning [73,74]. Nevertheless, it was thought for a period of time that extinction is synonymous to forgetting [75]. But this turned out not to be the case since forgetting is when a memory is completely erased or lost, whereas extinction is a process that is carried out repeatedly until complete inhibition of the expected response, as if it is learning of inhibitory type [67,76–78]. Besides, extinguished memories are not forgotten especially that they can recover spontaneously [67,76], or by changing the context, termed renewal [79,80], or by reinstating the US even without pairing to the CS, termed reinstatement [81].

1.3 PTSD

Posttraumatic stress disorder or PTSD is a devastating anxiety disorder that usually develops following the experience of a life-threatening event like harsh accidents, sexual abuse, combats, or natural disasters [82]. It is estimated that 1 in 8 individuals who experience a traumatic life event will develop PTSD [83]. Amongst the main hallmarks of this mental disorder are persistent and intrusive re-experiencing memories, nervous hyperarousal, and avoidance of trauma-related cues. Such symptoms interfere with the daily functioning of the patients afflicted with this disorder often to the point of physical and emotional disability [82]. Deregulation of the stress response is one of the main culprits for developing PTSD, also the formation of strong associations between the fear-inducing event and the cue or the context where the event took place is another [84].

Concerning treatment options for PTSD, the antidepressant class of selective serotonin reuptake inhibitor (SSRI) is considered the first choice in drug treatment. The response rates to this class, however, does not exceed 60%, where 20-30% of PTSD patients on SSRI-treatment fail to achieve full remission [85]. D-cycloserine (DCS) is a partial agonist to the NMDA (N-Methyl D-aspartate) receptors, and it has been shown that it facilitates extinction learning in rodents [86–88]. Another line of treatment is exposure-based therapy, where the patient is reminded of the traumatic episode while currently being in a safe environment in order for the patient to break the tie between the fear-eliciting cue/context and the trauma itself. During such treatment, other cognitive restructuring and anxiety management techniques are employed [89]. Of note, exposure therapy can occur in a real-life setting, but more recently also in virtual reality, and it has been shown that the combination of a cognitive enhancer like D-cycloserine and virtual reality exposure provide a promising treatment for chronic PTSD patients [90]. Moreover, there is another treatment method that proved to be effective for PTSD patients, it is the eye movement desensitization and reprocessing (EMDR) [91]. EMDR desensitizes patients to anxiety and integrates information processing through complex and specific desensitizing treatment method [92].

Functional neuroimaging were able to identify three brain regions that may be involved in the pathophysiology of PTSD. First, the amygdala (AMY) has been observed to be hyper-responsive during symptomatic states and is positively correlated with symptom severity of PTSD. Secondly, the medial prefrontal cortex (mPFC) appears to have diminished size, and is hypo-responsive during symptomatic states and the emotional cognitive tasks in PTSD. Thirdly, the hippocampus (HPC) was found to have reduced volume, as well as diminished neuronal and functional integrity in PTSD. All these findings lead to a hypothesized neurocircuitry model of PTSD which posits that the AMY is responsible for the fear symptoms the PTSD patients express due to its hyper-responsivity, whereas both mPFC and HPC – due to their hypo-responsivity and diminished functional integrity – fail to inhibit the AMY [93].

Chapter Two

2.1 Research Question

Taking up from where others left off.

2.1.1 Scope of work

The exposure to fear and traumatic events is, unfortunately, a common experience, and there is a high chance of developing an anxiety disorder because of that. However, little progress has been made towards identifying interventions and treatment strategies to alleviate trauma reactions and decrease rates of having an anxiety disorder [1]. Currently, the most effective treatment for anxiety disorders is exposure-based therapies, during which the patient is repeatedly confronted with the originally fear-eliciting stimulus in a safe environment so that the once fearful stimulus can be newly interpreted as neutral or safe [2]. The success of exposure-based therapies is mainly dependent on the reactivation of the traumatic memory [2], which may open a time-limited process called memory reconsolidation, where the retrieved memories during such period are believed to be susceptible to disruption [3,4]. Exposure-based therapies benefit from the memory reconsolidation window, where the patient can dissociate the tie between the context and the fear, and relearn that the context is no longer harmful or at least neutral [5].

It has been shown recently that the exposure therapy-like approaches, utilizing reconsolidation-updating paradigms, are quite effective to attenuate the response to fearful stimuli in humans [6], and in rodents [7]. It has been also shown that a similar paradigm could be even successful to prevent drug craving and its relapse in rodents [8]. During the period of memory reconsolidation, it is strongly believed that there is a learning process taking place and that it could be beneficial if pharmacological means are used to even enhance such process [9,10]. Histone deacetylase inhibitors (HDACis), are amongst the promising candidates that could permanently modify fearful memories [11] attributing their effectiveness to their direct effect on modifying chromatin compaction. Indeed, increments in histone acetylation could provide a stable and permanent effect on gene expression [12] which is a requirement for long-lasting forms of memory [13]. Also, epigenetic mechanisms in general can target a variety of nuclear processes involved in neuronal plasticity rather than being restricted to a particular signaling pathway [14].

Surprisingly, almost all of the studies aimed to attenuate fearful memories by using either behavioral or pharmacological approaches, have exclusively focused on recent (day-old) memories, without showing whether these approaches will also be effective for remote (month-old) memories. Since remote fear memories are persistent to disruption [15], therefore, there is a great need to explore options to overcome them.

Recently, Gräff and colleagues have shown that such reconsolidation-updating mechanisms alone are not sufficient to attenuate remote traumatic memories in mice. In particular, they found that whereas the recall of recent memories induces a limited period of hippocampal neuroplasticity mediated, in part, by S-nitrosylation of HDAC2 and histone acetylation, such plasticity was absent for remote memories. However, by using the HDAC2-targeting inhibitor (HDACi) CI-994 during reconsolidation, even remote memories could be persistently attenuated [16]. Thus, applying HDACis during memory reconsolidation might constitute a treatment option for remote traumata (Figure 2.1).

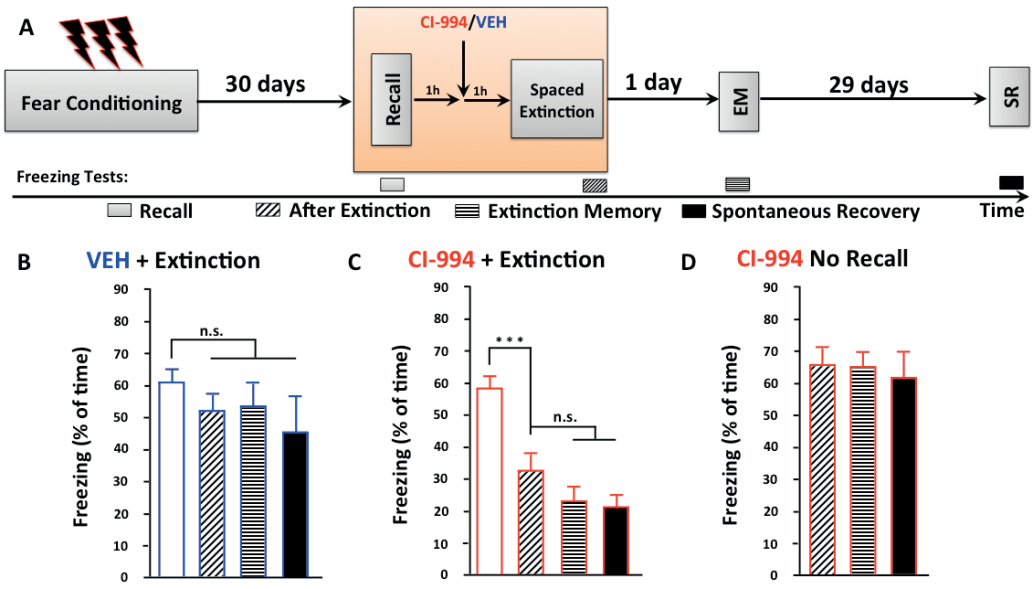

Figure 2.1: Remote memories become amenable to exposure-based therapy approaches with HDACi treatment. A) Schematic of the experimental setup. In spaced extinction, animals were exposed to the context 2 sessions per day 2h apart from each other. EM is the extinction memory test 24 hours after the last session of spaced extinction. SR is the spontaneous recovery test, where animals are tested for their retain of extinction memory 1 month following spaced extinction. B) In vehicle (VEH)-treated animals, remote fear cannot be extinguished. C) In HDACi CI-994-treated animals, remote fear was persistently attenuated and there were no signs of spontaneous recovery. D) The beneficial effect of the HDACi was only observed in the presence of the memory recall, as without it, the fear memory was not reduced [94].

2.1.2 Objectives

The current project aims at identifying which neuronal subpopulations are activated during successful memory attenuation, and what molecular processes are underlying successful memory extinction. For this, I have been using a combination of exposure-based therapies in transgenic mice, which allow for a time-limited activation of neurons upon remote memory recall, making it utterly possible to visualize those neurons. Further, I will also aim to determine their causal implication in successful memory extinction. To this end, I will be using a combination of pharmacological approaches in these transgenic mice, enabling us to selectively activate and/or deactivate those neurons and to assess the behavioral consequence of such manipulations. Finally, I will aim at identifying the molecular mechanisms that underlie such pharmacological intervention. Together, the projected results should yield for the first time insight into which neuronal subpopulations are causally involved in successful extinction or remote fear memories.

The overall goal of this project is to identify which neuronal subpopulations are activated during successful memory extinction, and what molecular processes are underlying successful memory extinction. Particularly, the specific objectives that will allow us to realize the overall goal are as follows:

Specific Aim1: ***Identification of the neuronal subpopulations that get reactivated during successful attenuation of remote fear memories***

Specific Aim2: ***Examining the loss-of-function of reactivating the recall-induced neurons for successful attenuation of remote fear memories***

Specific Aim3: ***Exploring the gain-of-function of reactivating the recall-induced neurons for successful attenuation of remote fear memories***

Specific Aim4: ***Investigating the molecular processes that underlie successful attenuation of remote fear memories***

2.1.3 Significance

Addressing the objectives of this project will allow us for the first time to:

i. Determine whether the original traumatic memory trace has been permanently updated or a new memory trace of reassurance/safety has been superimposed over the original one – a key open question in the field of brain research.

ii. To understand successful remote memory extinction at the molecular neurocircuit level, at a so far unachievable level of specificity.

iii. Use the obtained results as a template for future studies to quickly and reliably gauge the efficacy of interventional strategies against fear and related anxiety disorders, a clear benefit over existing behavioral methods with the same purpose.

2.2 Materials & Methods

The true method of knowledge is experiment.

- William Blake

1.Animals

Double transgenic TetTag lines (Fos-tTA, Fos-EGFP/tetO-lacZ, tTA*) and (Fos-tTA, tetO-tdTomato,-Syp/EGFP*) (Jax mice number 008344, and 012345, respectively) were used for the experiments. Both lines contain an inducible transgene, *lacZ* and *tdTomato* (tdT), respectively. The first double transgenic line with the *lacZ* transgene was used for all the experiments except for the sorting ones where the tdT expressing line was used. All mice had food and water ad libitum. They were raised on a doxycycline (DOX) rich diet (1g/kg pellets) from weaning. 3d prior to the session where the tagging window is desired, the DOX diet is replaced by a normal chow, and then provided back to the animal as soon as the tagging window is no longer needed. For the catFISH experiments, wild-type C57BL/6 mice were used. Mice were at least 10-12 weeks old at the start of the experiments, and they were all

males. All animal experimentations were done and approved under the veterinary cantonal authority in Switzerland.

2. Behavioral paradigms

Contextual Fear Conditioning (CFC). Animals were acclimatized for 2 days to handling several times a day. Contextual fear conditioning training consists of a 3-min habituation of mice to the conditioning chamber (TSE systems) followed by three 2 s foot shocks (0.8mA) with an intertrial interval (ITI) of 28 s. After the shocks, the animals remained in the chamber for an additional 15 s. 30 d later (spent in the home cage, during which animals were monitored for their overall health), the spaced fear extinction paradigm was carried out (Figure 3.3 A).

Spaced Extinction. Mice were re-exposed to the same chamber for 3 min without receiving the foot shock (to recall the memory), and returned to their home cage for 1 h, after which they were once again exposed to the training chamber for 3 min. This procedure was repeated on three subsequent days, for a total of 4 d of spaced extinction. In experiments where the tagging window was opened by switching off the DOX, the mice were exposed to the recall session on the first day, and then started the spaced extinction sessions the following day. This is to ensure that the first session of extinction will not elicit transgene expression if performed 1 h after the recall. In between both sessions of spaced extinction mice were injected with CI-994 (an HDAC2-targeting inhibitor – 10 mg/kg i.p. dissolved in 10% dimethyl sulfoxide, 30% cremophor (Sigma-Aldrich), and 60% 0.9% normal saline). This drug has previously been shown to enhance successful remote fear extinction [94]. The CI-994 was given in all the experiments except for the DREADD hM3Dq ones. This is in order to be able to see the effect of the CNO activation to the hM3Dq if there is one. Otherwise the CI-994 might conceal this effect. For the Daun02 experiments, the Daun02/VEH were infused 90 min post-recall, and the spaced extinction was performed as described earlier, while for the DREADD experiments, the spaced extinction was performed as described 30 min following an i.p. injection of the CNO or its VEH.

Massed Extinction. This variation of fear extinction was only used in the catFISH experiments. The animals were re-exposed to the same chamber for 3 min without receiving the foot shock (to recall the memory), and returned to their home cage for

one hour, after which they were once again exposed to the training chamber for a total of 18 min. No CI-994 was given to the cohorts of this experiment. The freezing readout in this experiment is plotted between the recall session and the last 3 minutes of the 18-minutes massed extinction as shown in (Figure 3.9 B).

Open Field Test. The open field (OF) arena was 72 x 72 cm in dimension, and was defined mainly into 3 different zones: the centre, the periphery, and the intermediate. It was used to measure anxiety and exploration as well as locomotion. The animals were handled several days before being placed inside the arenas, which they were left for 20 minutes to explore. After that they were carried back to their home cages. The data was recorded with a camera, and a special software (EthoVision XT, Noldus) was used to measure the time spent by the animals in each of the 3 zones, their velocities, and the distance moved as well.

3. Immunohistochemistry

Animals were anaesthetized using sodium pentobarbital (150 mg/kg, i.p.; <200mg/ml), then transcardially perfused using the paraformaldehyde (PFA) 4% for 10 minutes, and brains removed and kept in PFA 4 % overnight at 4°C, and then replaced by 30% sucrose until sectioned using a vibratome (VT1000 S, Leica biosystems). The brains were sliced at 35-40 μm free floating coronal sections, and washed in PBS 0.1 M for 3 times 10 min each, and blocked in 1 % BSA, and incubated for 4 nights with primary antibodies. 4 d later, the slices were washed with PBS 0.1 M with 0.1 % Triton-X, and then the fluorescently conjugated secondary antibodies were added for 2 h at room temperature (RT) in a dark place with constant gentle shaking. Secondary antibodies were washed in phosphate buffer 0.12 M for 3 times 10 min each, and then the slices were mounted on a slide using a mounting media with the nuclear stain Hoechst 33342 (ThermoFisher, Life Technologies). Slides were kept in a dark at 4°C until image acquisition. Antibodies used were as follows: βgal (1:500 dilution, AHP1292 abD Serotec, rabbit), cFos (1:1000 dilution, Sc-52, goat), GFAP (1:1000 dilution, clone G-A-5 Sigma, mouse), CamKII (1:300 dilution, clone 6G9 Cayman 1011437, mouse), GAD67 (1:500 dilution, MAB5406, mouse), AcH3K9 (1:500 dilution, ab12179, mouse), secondary antibodies conjugated with Alexa fluorophore 488, 568, and 647 were used (1:1000 dilution).

4. Cellular compartment analysis of temporal activity by fluorescence *in situ* hybridization (catFISH)

For this experiment, wild-type animals were used as previously indicated. The mice were CFC trained, and tested for the memory 30d later. 45 min following the recall session, the animals underwent a massed extinction that lasted 18min. Animals were immediately sacrificed by neck dislocation, and their brains were extracted and snap frozen directly. In parallel, a control group -to account for the basal level of activation due to home cage activity- was taken out of its home cage and sacrificed straightaway. All brains were cut with a cryostat (CM 3050S, Leica Biosystems), and the coronal slices were attached on charged super frost plus slides (Thermo Fisher). The *in situ* hybridization was carried out by the EPFL histology core facility (HCF) following the protocol of the manufacturer of the RNA probes (RNAscope, ACDBio) used in this experiment. Two RNA probes were used against immediate early gene (IEG) markers, namely; Homer1a (H1a), and cFos. H1a probe was conjugated with Cy2 fluorophore, whereas the cFos probe was conjugated with Cy3. For each slide used for catfish, an internal control was performed to detect the housekeeping gene Ppib and the bacterial gene Dapb, which served as positive and negative control respectively.

5. X-gal staining

After trying the X-gal staining protocol on the free floating coronal sections for the PFA perfused brains, the results were not satisfying, so I optimized the protocol to the following: Animals were anaesthetized using sodium pentobarbital (150mg/kg, i.p.), and then transcardially perfused with PBS 0.1M very briefly to remove any blood from the brain tissue. The brains were extracted and snap-frozen using liquid nitrogen and isopentane. The frozen brain were sliced coronally (20 µm) using a cryostat (CM 3050S, Leica Biosystems), attached on charged slides (superfrost plus, ThermoFisher), and kept at -80°C until further processing. Prior to processing, the slides were allowed to thaw and the slices fixed using glutaraldehyde 2 % for 10 min at room temperature (RT). Slides were washed in PBS 0.1 M 3 times for 10 min, equilibrated with X-gal reaction buffer solution (0.24 M PBS, 2 mM $MgCl_2$, 0.02 % IGEPAL, 0.01 % sodium deoxycholate) for 10 min at RT, stained with the X-gal

staining solution (X-gal reaction buffer solution, 0.5 mg/ml X-gal, 3ul/ml nitroblue tetrazolium) for 1 h at 37°C, and washed in PBS for 5 min, 3 times. Counterstaining was done with nuclear fast red for 5 min at RT, followed by washing in dH_2O for 5 min. Then, tissue dehydration steps using different concentrations of ethanol were performed: 70 % for 2 min, 90 % for 2 min, and finally with 100 % for 2 min. Slides were immersed in xylene for 2 min, and then coverslipped with Eukitt mounting medium and stored at RT before being imaged (Leica DM 5500 microscope, bright field).

6. Image acquisition and quantification

For Immunolabelling. Images were acquired using a Zeiss LSM700 laser scanning confocal microscope. The settings for the acquisition (laser intensities, and gain) were the same across samples. Regions were acquired with the tiling option using a 20X objective and a Z-stack with 2 μm interval steps. Typically, 4 to 5 images per region per animal were quantified by a person unaware of the experimental condition of the animal. After using negative controls to setup the thresholds, the quantifications were carried out manually on the inner most layer of the stack. If in doubt about a certain signal, one layer upwards or downwards in the Z-stack was verified for the authenticity of such signal. The channels were quantified separately using Fiji. Each signal was quantified in its own channel using the "cell counter" plugin in Fiji adding a marker on every quantified cell, and in the end all layers are merged together and a cell is to be considered double positive if the quantification markers from the separate channels overlap. The overall amount of cells in the region was quantified in the Hoechst channel using the ROI tool, i.e., calculating the average amount of cells of the whole region by knowing the area of the whole region and the area of a single cell. This method has been strictly verified comparing the results of this method and the meticulous manual counting of every single cell in the region, and the results were practically identical. The rates were calculated according to the formulas below. The images used to define the identity of the reactivated population were acquired using the 40X oil objective.

$$Activation\ Rate = \left(\frac{\beta gal + cells}{Hoechst + cells}\right) \times 100$$

$$Learning\ Rate = \left(\frac{cFos + cells}{Hoechst + cells}\right) \times 100$$

$$Extinction\ Rate = \left(\frac{cFos + cells - (\beta gal/cFos + cells)}{cFos + cells}\right) \times 100$$

$$Reactivation\ Rate = \left(\frac{\beta gal/cFos + cells}{\beta gal + cells}\right) \times 100$$

$$Reactivation\ Rate\ chance\ level = \left(\frac{\beta gal + cells}{Hoechst + cells}\right) \times \left(\frac{cFos + cells}{Hoechst + cells}\right) \times 100$$

The quantifications for the AcH3K9 antibody and immunocytochemistry of the DREADD experiment *in vitro* were carried out by densitometric analysis where the signal of either AcH3K9 or the cFos (for the first and second experiments, respectively) was identified in terms of fluorescence intensity, and with the following formula for the corrected total cell fluorescence (CTCF) [95,96]. CTCF = Integrated density – (area of selected cell X mean fluorescence of background). This corrected value was used as either an arbitrary unit of fluorescence in the AcH3K9 experiment, or as fold increase compared to the control's intensity in the DREADD one.

For catFISH. Images were acquired using a Zeiss LSM700 laser scanning confocal microscope. In this experiment, four different brain slices from 5 different animals were quantified. The images were acquired in frame mode with a frame size of 512x512 pixels using a 40x oil-immersion objective. The cells were counted - by a person blind to the identity of the slides - with the cell counter plugin of Fiji. H1a and cFos+ cells were quantified in their corresponding separate channels, and then both channels were overlapped with their markers to be able to identify the double positive population. The rates were calculated according to the formulas below.

$$Activation\ Rate = \left(\frac{cytoplasmic\ H1a + cells}{Hoechst + cells}\right) \times 100$$

$$Learning\ Rate = \left(\frac{nuclear\ cFos + cells}{Hoechst + cells}\right) \times 100$$

$$Reactivation\ Rate = \left(\frac{cytoplasmic\ H1a/nuclear\ cFos + cells}{cytoplasmic\ H1a + cells}\right) \times 100$$

7. Cannula Implantation and cannula-mediated Daun02 infusions.

Cannula implantation. 1-2 weeks following the CFC training, animals were put to sleep in deep anesthesia using an induction mixture composed of "triple-shot" mix of Fentanyl (0.05 mg/kg) + Midazolam (5 mg/kg) + Medetomidin (0.5 mg/kg). Brief shaving of the scalp was followed by a subcutaneous injection of local anesthesic lidocaïne (6 mg/kg) and bupivacaine (2.5 mg/kg) along the line where the incision will take place. 5 min later a small skin incision was made, after which the animal was placed on a heating pad - adjusted to maintain constant body temperature - into the stereotaxic frame, and the body temperature was monitored using a rectal thermometer. An eye gel (Viscotears, Alcon – Novartis) was applied on the eyes throughout the surgery. The scalp was disinfected with Bedatine and a small incision was made to expose the cranium. The surface of the cranium was cleaned using 10 % hydrogen peroxide (Sigma-Aldrich) to bleach the surface of the cranium enabling clear visualization of lambda and bregma. Afterwards, the cranium was perforated with a 30-gauge drill using the following coordinates: -2.0 mm anterior–posterior, ±1.5 mm medio–lateral from Bregma to target the DG in the dorsal hippocampus, whereas for the CA3 I used -1.68mm anterior-posterior, and ±2.05 mm medio-lateral. Any debris due to drilling was removed using cotton buds soaked with PBS. Then, the bilateral guide cannula was placed on the stereotactic holder and carefully inserted into the drilled holes. The width of the guide cannula was 3.00 mm center-to-center, and was cut 1 mm below pedestal (which makes it 1.00 mm in total in Z-axis). Application of a light cured glue (iBOND Total Etch, Heraeus and Tetric EvoFlow

Filling material, Ivoclar Vivadent) around the cannula and exposing it to 5-10 seconds pulse of the light curing LED (Elipar s10 Led, Curaden) was used to dry the glue. After that, the guide cannula holder was unscrewed carefully and removed from the guide cannula, the dummy cannula inserted into the guide cannula and covered. The incision was closed with absorabable vicryl 4.0 sutures. Post-surgery, the animals received a subcutaneous injection of anesthesia reversal mixture ("triple-shot" mix of Naloxon (1.2 mg/kg) + Flumazenil (0.5 mg/kg) + Atipamezol (2.5 mg/kg)) followed by a subcutaneous injection of previously warmed (37°C) physiological NaCl. The animals were then placed on a heating pad to wake up while monitored for recovery signs. The overall health of the animals that underwent surgery as well as their incision marks was carefully monitored. Mice are used in experiments not before 10-14 d after the surgery. Only animals with cannula traces at the targeted region were considered for histological, behavioral, and statistical analyses.

Cannula-mediated infusions. Mice were briefly anaesthetized using Isoflurane (3-4%), and the internal/injection cannula (with a dorso-ventral coordinates of 2.00mm) was inserted instead of the dummy. The drug infusion took place at a flow rate of 0.5 ul/minute, leaving the injection cannula in place for 1-2 more minutes to prevent leakage to other areas. The dose of Daun02 injected is a total of 2μg in 1ul per hemisphere. The Vehicle used for the control groups was artificial cerebrospinal fluid (ACSF).

8. DREADD experiments

In vitro. 2 viral constructs were used to infect rat primary culture neurons. pLVX-hM3Dqcherry-IRES-LacZ, and pLVX-hM3Dqcherry-IRES-Zcal (for control experiments). The primary culture neurons were plated on a poly-L-lysine coated plates (35mm - $0.3X10^6$ cells per dish). The cells were fixed for immunolabelling after 1 h of CNO (30 μM) treatment, or after 2 h of CNO and Daun02 (45 nM) treatment. The increase in cFos fluorescence was determined through densitometric analysis of signal acquired using Fiji.

In vivo. 1 week following the CFC training, animals were anaesthetized and prepared for surgery as described above. The following coordinates were used: Animals received bilateral craniotomies using a 30-gauge drill bit at -2.0 mm anterior–

posterior, ±1.3mm medio–lateral, and -2.0mm dorso-ventral for dentate gyrus injections. All mice were injected with 250 nl of AAV9 virus (AAV_9-TRE_{tight}-hM3Dq-mCherry) at a controlled rate of 0.1 ul/min using a mineral oil-filled to a 10 ul Hamilton microsyringe (1701 RN neurosyringe) in a microsyringe pump (UMP3; WPI). The needle was slowly lowered to the target site. The needle remained at the target site for another 5 minutes post-injection before being slowly withdrawn. The animals were monitored after surgery, and the transgene was allowed to be expressed for 21d before proceeding with the behavioral experiments. The CNO was prepared in physiological saline (0.9%), and was injected to the animals (3 mg/kg i.p.) 30min before the first extinction session on 4 consecutive days.

9. Cell-dissociation and Fluorescence Activated Cell-Sorting (FACS)

One hour after the last extinction session, animals from the TetTag line expressing the EGFP and tdT transgenes were sacrificed and brains were extracted quickly in cold Hank's balanced salt solution (HBSS) (Gibco, Invitrogen). The HPC was promptly extracted, minced into 1 mm pieces and placed into the gentleMACS C tube pre-filled with enzyme mixes from Miltenyi Biotec adult brain dissociation kit. The tube was then attached upside down onto the sleeve of the gentleMACS Octo Dissociator (Miltenyi Biotec) with Heaters. The gentleMACS Program 37C_ABDK_01 for brain tissue dissociation was carried out, and after 30 min the cells were collected at the bottom of the C tube with a gentle centrifugation. The sample then was resuspended in HBSS and applied to a 70 μm cell-strainer on a 50 mL falcon tube to remove the debris. 1 μL of the fluorescent cell viability indicator propidium iodide (PI) (Fluka) was added to the sample, and 1 ul of Hoechst (ThermoFisher, Life Technologies) as well. A final filtration step was performed with the 70 μm cell-strainer before proceeding to the cell-sorter (BD FACSAria II flow cytometer). Gatings were performed based on the single events/cells and then based on the signals of Hoechst and PI. And the final gates were based on the fluorescence of the EGFP and tdT. Cells were collected in 384-well plates - provided by our collaborators - according to which population they belong to (double negative for EGFP, and tdT; EGFP positive; tdT positive; and finally double positive for EGFP

and tdT). Some extra cells were collected in RNase-free microfuge tubes (Thermo Fischer) after filling all the wells of the plates to control the quality of the collected cells by extracting and evaluating the quality of the RNA. From pooling 2-3 hippocampi of similarly behaving mice, and after finishing the sorting for the 384-well plates, the sorted bulk in tubes was as follows: [double negative population: ca. 13,000 cells; EGFP positive population: ca. 700 cells; tdT positive population: ca. 160 cells; EGFP/tdT double positive population: ca. 150 cells].

Chapter Three

3.1 Results

Finding out if the waiting was worthwhile!

1. Inspecting the behavioral readout of a double transgenic mouse line

Before addressing the main aims of the project, I decided to scrutinize the effect on the behavior of the two foreign genes present in the mouse line I will use throughout the entire project, viz. the TetTag (Figure 3.1) mouse line [19]. And despite it has been extensively characterized in Mark Mayford's lab, there is always a chance of disparate behavioral outcome between labs. Therefore, I designed a simple pilot experiment to compare the behavior of animals from this line with wild-type (WT) animals of the same genetic background, and assessed their level of anxiety and overall exploratory behavior in an open field test (Figure 3.2). The results showed no difference between both cohorts: TetTag mice show similar exploration patterns to the different zones of the OF arena as measured by the distance moved by the animals in the three zones of the arena (Figure 3.2 A-D), and the time spent there (Figure 3.2 E-H), and – finally – their velocities in each of these zones (Figure 3.2 E-H).

Next, I proceeded to examine whether the TetTag animals are able to acquire an associative fear memory, then store it for a long time to express afterwards, and later attenuate following an extinction paradigm. To this end, the animals underwent contextual fear conditioning (CFC) training as a learning paradigm to assess their degree of learning for both, fear and afterwards extinction, upon remote fear recall (Figure 3.3 A). The animals showed a high degree of learning measured by the prominent freezing they have produced upon a recall test 30 days following the acquisition (Figure 3.3 B). Afterwards, a spaced extinction paradigm was performed on these animals, where they were exposed once again to the context they have received the shock at, but this time without the shock. Two sessions were performed per day for four consecutive days that end by reduction of the fear as measured by lowered freezing compared to that during the recall day. The fear was significantly attenuated following the spaced extinction (Figure 3.3 B). And to assess the degree of extinction, a Δ freezing (or a freezing differential) was developed by calculating the difference in freezing between the recall session and the last extinction session (Figure 3.3 C). The higher the Δ freezing, the better the quality of fear extinction. However, and upon plotting the individual Δ freezing of animals, there was a strong disparity allowing their classification into good, and bad extinguishers. Such classification was based on their Δ freezing, where good extinguishers were those scored positive values, whereas bad extinguishers were those scored zero or negative values (Figure 3.3 D).

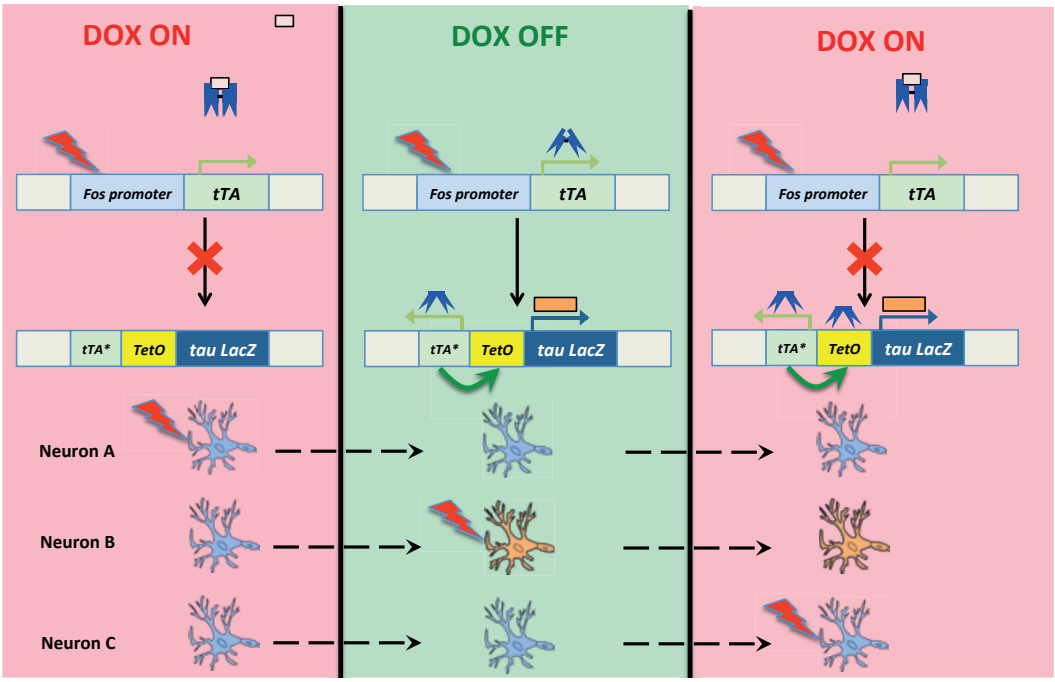

Figure 3.1: Double transgenic TetTag system. Tagging of activated neurons is achieved by two transgenes present in the TetTag mouse line. Left block, the mice are raised on food containing doxycycline (DOX). While DOX is on, any neuronal activation (indicated by red lightning-bolt symbols) driving the expression of tTA [tetracycline transactivator transcription factor] through cFos–promoter activation will not trigger tagging. DOX in this case is blocking the activation (indicated by the red "x") of the TetO promoter (like in neuron "A"). Middle block, the time window for tagging can be opened by switching mice to food without DOX. Neuronal activation will now activate the transcriptional feedback loop and start expression of tauLacZ (like in neuron "B"). Right block, the time window is closed again by putting mice back on DOX to block further feedback loop activation (like in neuron "C"). However, neurons that were activated during the "OFF DOX" time window will continue to express tauLacZ (like in neuron "B"), because the feedback loop can maintain its own activation through the doxycycline-insensitive tTAH100Y (tTA*), where H100Y represents a point mutation in the His100→Tyr100 (Adapted from Reijmers et al., 2007 [97]).

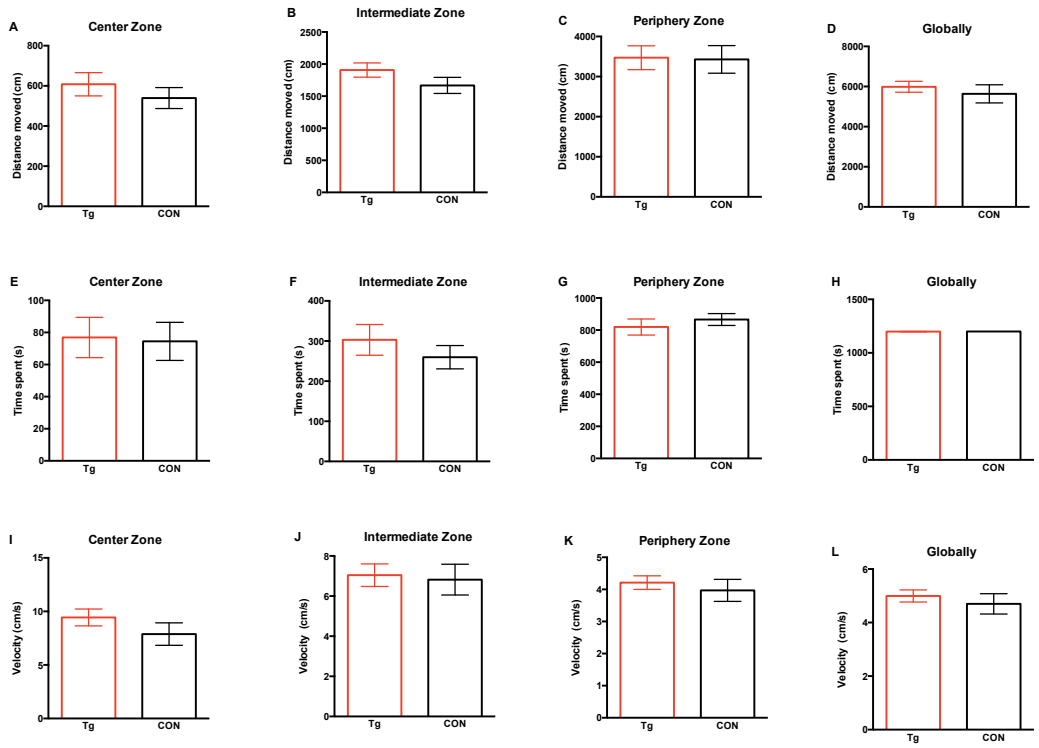

Figure 3.2: Open field test shows no difference between TetTag and WT animals. (A-D) Distance moved by the animals in different zones of the OF arena. **(E-H)** Time spent by the animals in different zones of the OF arena. **(I-L)** Velocity of animals in different zones of the OF arena. Means ± SEM are shown in (A) to (L), compared with two-tailed, unpaired *t*-test. [Tg: Transgenic TetTag group; CON: wild-type control group].

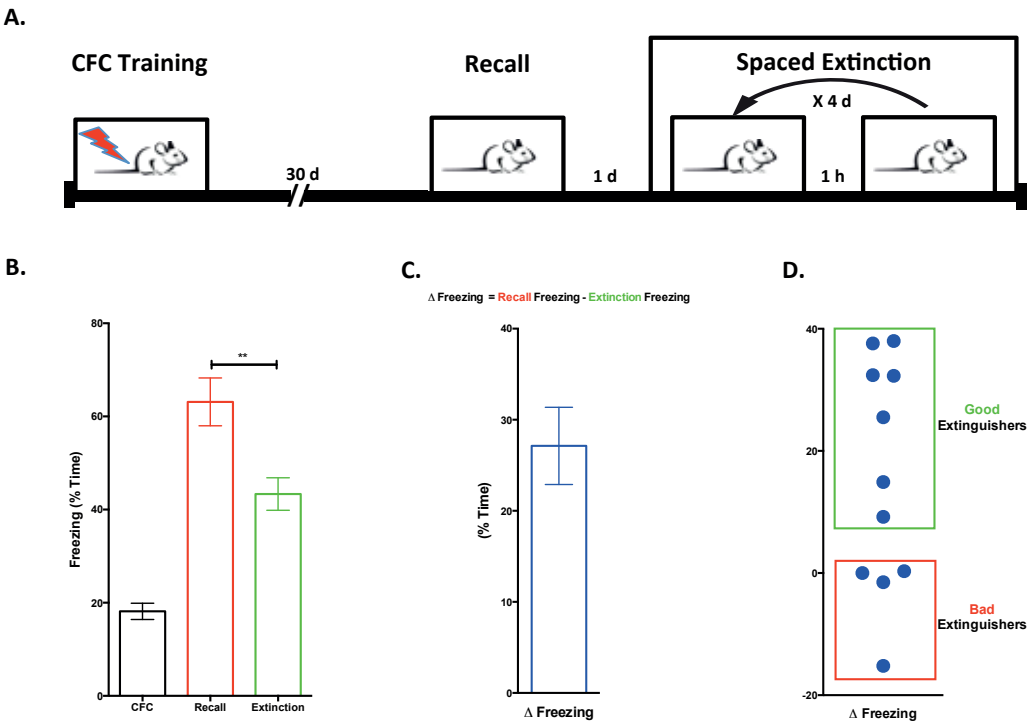

Figure 3.3: TetTag animals show high freezing during the recall of remote fear, which is amenable to extinction training. (A) Timeline of experimental design. Briefly animals were contextually fear conditioned, and after 30d, the recall session was performed. 1d later, the spaced extinction started (two sessions per day) and spanned 4 consecutive days. **(B)** The difference in freezing between the three epochs of training, recall, and last extinction session shows a high freezing during the recall which can be significantly reduced after a spaced extinction paradigm (*n=8, **P=0.006,* compared with two-tailed, unpaired *t*-test). **(C)** The Δ freezing offers another representation of the relation between the freezing during the recall and extinction [data shown for the same group used in (B) *(n=8)*]. It provides an idea of how well the animals extinguished their fears. **(D)** The Δ freezing results of individual animals show clear variation between animals where they can be classified into good (Δ freezing > 0), and bad extinguishers (Δ freezing ≤ 0) (*n=11*). Means ± SEM are shown in (B) and (C). [CFC: contextual fear conditioning].

2. Visualising the activated neuronal populations upon remote fear memory recall and following fear extinction

After validating the efficiency of the TetTag line in learning fear as well as attenuating it following extinction, I use their doxycycline-controlled window of labelling to address the first aim of my research question and identify the neuronal populations that are activated upon remote memory recall, and following memory extinction. It is noteworthy that the results of this section were reproduced in two different behavioural setups, but for the sake of consistency the data shown here come only from the most recent experiments conducted using the lab's setup. Hence, the data –down here - describe the behavior and immunohistochemistry of the very same cohorts. The following subsections are explaining how.

A. Design and behavior

Three TetTag cohorts were used in this experiment: CS/US-Recall, CS-Recall, and CS/US-NO Recall (Figure 3.4 A-C). All three groups were kept on doxycycline (DOX) containing food pellets. During the CFC training all the groups were on DOX, and 25 days later the DOX-containing pellets were changed to normal chow. Three days after removal of DOX, the recall session was performed for the CS/US-Recall group (Figure 3.4 A) to identify the population activated during remote fear recall. Whereas the other 2 groups serve as a control to assess the level of transgene expression as a result of the activation due to the recall of the context alone (CS-Recall, Figure 3.4 B), and to define the basal level of expression due to the home cage activity since the DOX was removed 3d before the recall and this could elicit unspecific labelling (CS/US-NO Recall, Figure 3.4 C). After the recall session, the animals were returned to the DOX-containing food pellets to proceed through the spaced extinction sessions (2 sessions for 4 days) not capable of producing further labelling as a result of this extinction paradigm. The freezing behavior of the animals was recorded every session, and 1h after the last extinction session, the animals were transcardially perfused with paraformaldehyde 4% to perform immunolabelling on the extracted brains. The freezing of the CS/US-Recall group was prominent and it significantly abated following extinction, whereas the CS-Recall group showed a

subsided freezing during the recall and no difference following extinction (Figure 3.4 D).

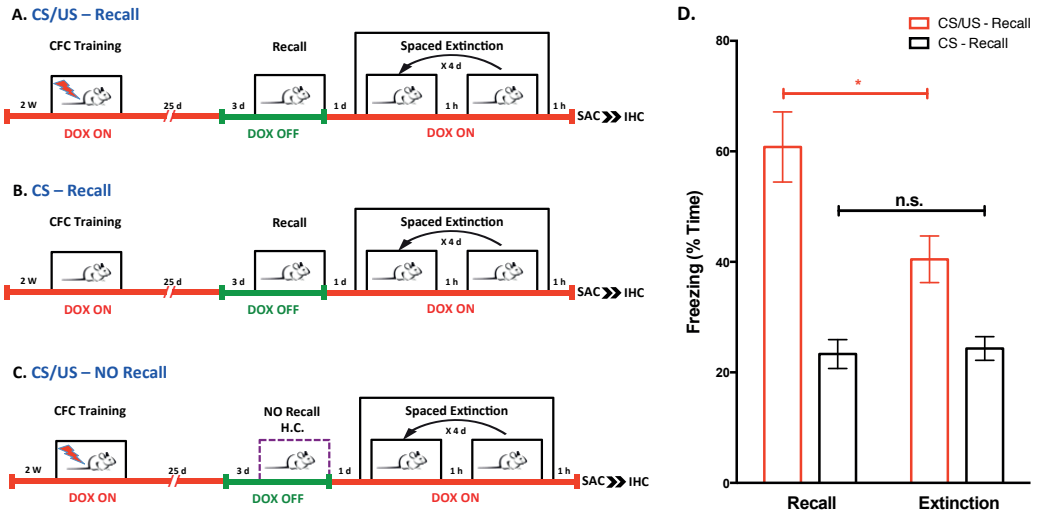

Figure 3.4: Three different TetTag cohorts used to study the fate of the recall-activated population upon successful fear attenuation. (A-C) Timeline of the experimental design of the three TetTag cohorts. **(A) CS/US-Recall**, where the animals receive CFC training and a recall session while off DOX, and a full extinction paradigm while on DOX. **(B) CS-Recall**, where the animals do not receive any shock during the CFC training and a recall session while off DOX, and proceed like in the previous group. This group serves as a control for the basal level of activation due to the context itself. **(C) CS/US-NO Recall**, where the animals receive CFC training and then they never receive a recall session, instead they stay in the home cage off DOX for the same period like the first group. This group serves as a control for the basal of activation due to the home cage activity. **(D)** The behavioral readout of the first two groups, where only the group that receives the shock shows a significant increase in freezing during the recall that can be significantly reduced following extinction (*CS/US-Recall, n=7, *P=0.0386; CS-Recall, n=10*). Means ± SEM are shown in (D), compared with two-tailed, unpaired *t*-test. [CFC: contextual fear conditioning; H.C.: home cage; SAC: sacrifice; IHC: Immunohistochemistry].

B. Labelling pattern

To reveal the populations activated during the remote fear recall and following the last extinction session, an immunolabelling was performed. The recall-activated population expressed the βgal persistently during the window of transgene expression when DOX was off of the animal's diet, whereas the population activated during the last session of extinction was labelled with the immediate early gene (IEG) marker cFos. The βgal antibody elicits a diffused pattern of cytoplasmic labelling, while the cFos antibody is nuclear (Figure 3.5). The Hippocampus (HPC) showed the highest

produced signal of labelling especially in the DG and the CA3 field (Figure 3.5), whereas the labelling at the CA1 field was daunting due to the inconsistent and often times unclear, i.e. diffuse pattern of staining for both antibodies there. For that reason, and for the rest of my project, I decided to focus on the DG and CA3 regions since they had the clearest reliable labelling signals.

C. Nature of activated populations

In an attempt to discover the nature of the populations that are activated at the recall and extinction sessions, a co-immunolabelling was performed to identify which marker these activated populations will co-label with. Firstly, the astrocytic marker GFAP showed that the activated populations are essentially neurons due to the lack of overlap between this marker and the activated populations (Figure 3.6 A – uppermost panel). Secondly, the majority (more than 95%) of the populations activated separately during the recall or the extinction co-localized with CamKII excitatory neuronal marker, whereas very scant amount of cells in both populations co-localized with the GAD67 inhibitory neuronal marker (Figure 3.6 B). Finally, the nature of the cFos/βgal double labelled population that represents the population that was active during the recall and extinction turned to be mainly (ca. 90%) excitatory as shown by the co-localization with the CamKII, however, there were very few cells (ca. 6%) of this population which overlapped with the GAD67 marker (Figure 3.6 A, and B).

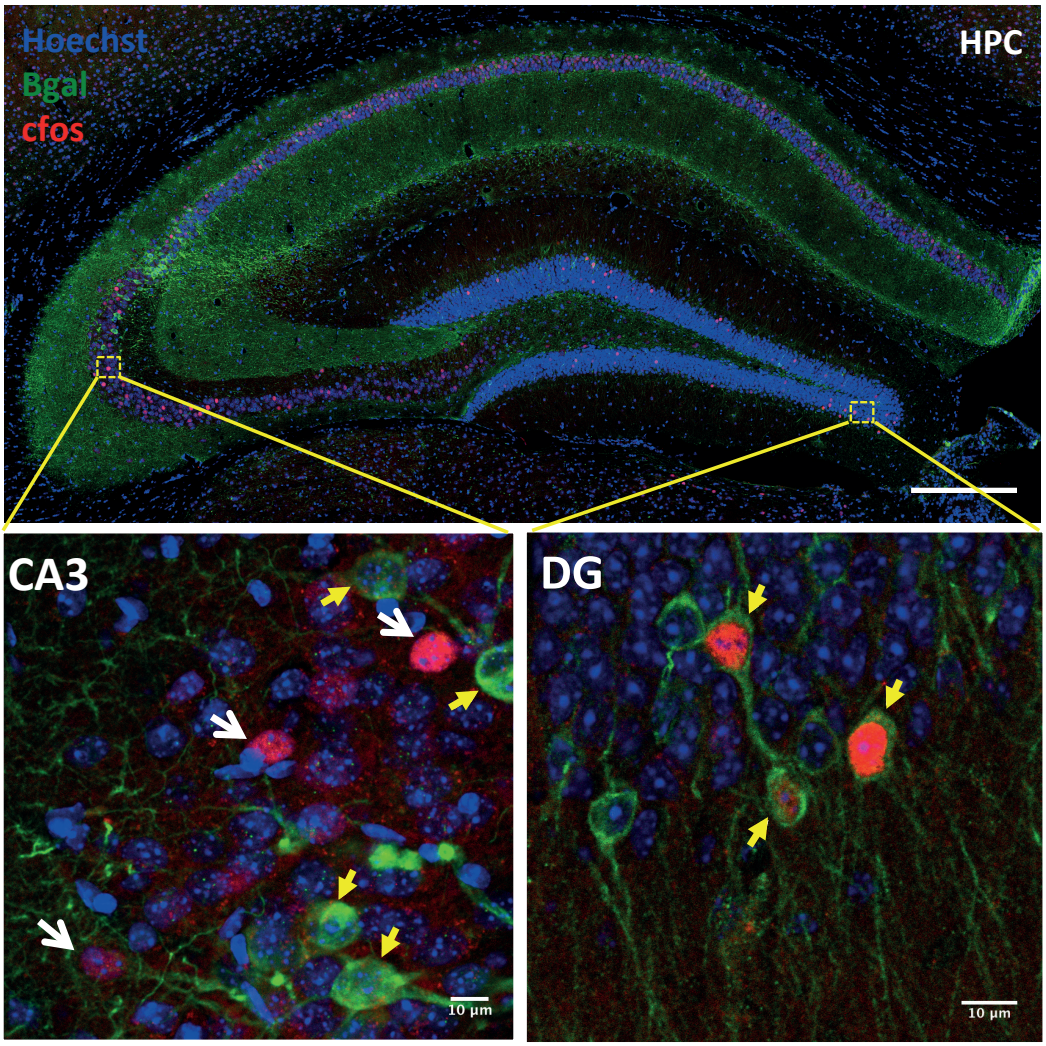

Figure 3.5: The hippocampus showed a degree of reactivation to the recall-activated population during fear extinction. The upper panel is showing an overview of the hippocampus and how the immunolabelling looked like, where the cell nuclei are stained with Hoechst in blue, the βgal in green, and the cFos in red. Scale bar, 300 µm. The lower panels show an enlarged area of the dashed yellow squares. In the DG (lower right panel) some cFos/βgal doubly labeled cells (solid yellow arrows) are observed compared to the CA3 region (lower left panel) where many single cFos cells (opened, white arrows), and single βgal cells (closed, yellow arrows) can be observed. Scale bars, 10 µm. [HPC: Hippocampus; CA3: CA3 field; DG: Dentate gyrus].

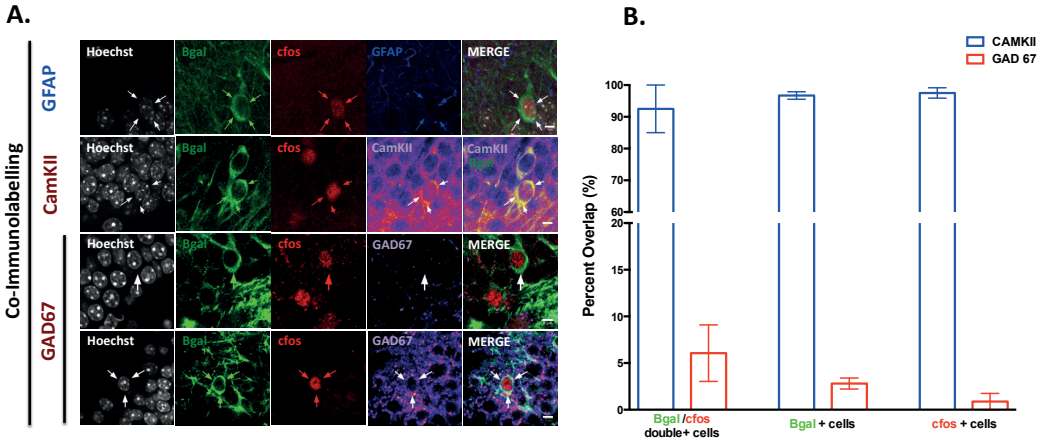

Figure 3.6: The recall-induced reactivated population mainly co-localizes with excitatory neuronal marker in the HPC. (A) Immunohistochemistry of cFos/βgal doubly labeled cells (solid arrows pointing at in the merge channels) in the HPC show no co-localization with the GFAP astrocytic marker, but very high co-localization with the excitatory neuronal marker CamKII, and very few cells co-localize with the inhibitory neuronal marker GAD67. In the separate panels, Hoechst (in grey) marks the cell nucleus, βgal (in green) marks the recall-activated population, cFos (in red) stains the activated cells during extinction. The fourth channel in the panel is in blue (for GFAP), and in fire for (CamKII and GAD67). The upper GAD67 panel shows no co-localization, yet very few cFos/βgal doubly labeled cells show co-localization with GAD67 (lower panel). Scale bars, 5 µm. **(B)** Quantification of the co-localization stainings performed on the cFos/βgal doubly labeled cells, as well as the single-labelled cells of βgal or cFos (*different animals analyzed n=4; cFos/βgal double labeled cells analyzed, n=48; βgal single labeled cells analyzed, n=446; cFos single labeled cells analyzed, n=59*). Means ± SEM are shown in (B), compared with two-tailed, unpaired *t*-test.

D. Quantifications and correlations

Advancing towards collecting more information about the activated populations in the HPC, I started by defining some parameters – summarized in the following bullet points - that will help me make sense of all the quantified signals in the HPC. All formulas used in quantification are described in the materials and methods section.

- Firstly, the ***activation rate***: to determine how many cells were activated during the recall of remote fear memory. The activation rates for both DG and CA3 were between 12-13% and were significantly higher than the control groups' activations due to the recalling of either the context only, or the basal activity in home cage (CS-Recall, and CS/US-NO Recall groups, respectively) (Figure 3.7 A).
- Secondly, the ***learning rate***: which reveals the overall amount of cells that were activated during the last session of extinction i.e. the learning population. The learning rates of the DG and CA3 were not significant from those of the control groups, and were between 2-2.5% (Figure 3.7 B).

- Thirdly, the ***extinction rate***: highlights the subset of the learning population that was not active during the recall. The extinction rate of the CA3 (ca. 93%) was significantly higher than that of the DG (ca. 76%) (Figure 3.7 C). This shows that the majority of the learning population at the CA3 was not active before during the recall, yet for the DG it appears that almost the quarter of the leaning population was active before during the recall.
- Finally, the ***reactivation rate***: to determine the amount of cells that were active during both epochs: the recall and the extinction. The reactivation rate revealed that the DG contains significantly two-fold higher amount of cells that were active during both sessions (ca. 3%) when compared to that of the CA3 (ca. 1.5%) (Figure 3.7 D). Upon ruling out the degree of reactivation due to mere chance, the normalized reactivation rate of the DG is still significantly higher than the chance level (ca. 1.6 fold), whereas the normalized reactivation of the CA3 is not (Figure 3.7 E).

Next, and since the amount of reactivated population at the DG was significantly higher than being just from chance, I wondered if it will carry any correlation with the Δ freezing. It turns out that the reactivation of the recall-induced population at the DG correlates positively with the Δ freezing, where the animals that had the highest amount of reactivation elicited the best quality of fear extinction – as reflected by their prominent Δ freezing - and vice-versa. However, this is not the case at the CA3 (Figure 3.8 A). Moreover, and to see how fear extinction might be affected by the number of cells activated exclusively during the extinction session, I checked the relation between the extinction rate and the Δ freezing to find out that at the DG there is a weak but significant negative correlation, yet for the CA3, there is not (Figure 3.8 B).

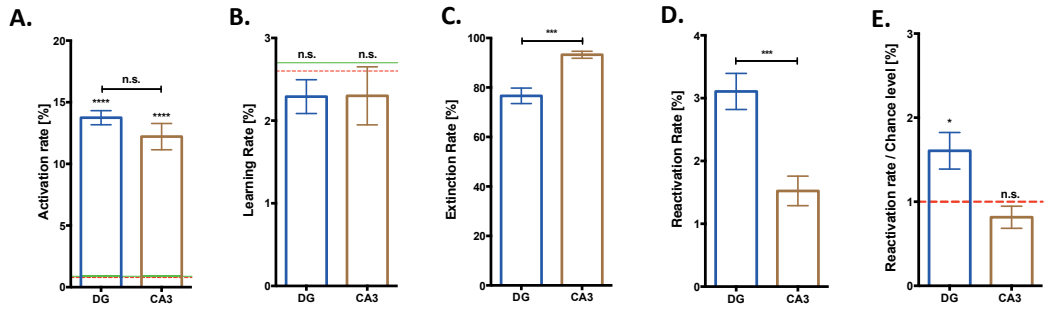

Figure 3.7: Quantification of the distinctly activated neuronal populations during the remote fear recall and extinction. (A) The activation rate of the DG and CA3 is significantly higher than the activation rates of the control groups (CS-Recall group, green solid line; CS/US- NO Recall group, red dashed line) (*DG, n= 7; CA3, n=9; ****P<0.00001*). **(B)** The learning rate of the DG and CA3 is not significantly higher than the learning rates of the control groups (CS-Recall group, green solid line; CS/US- NO Recall group, red dashed line) (*DG, n= 7; CA3, n=9*). **(C)** The extinction rate of the CA3 is significantly higher than that of the DG (*DG, n= 9; CA3, n=9; *P=0.0002*). **(D)** The reactivation rate of the DG is significantly higher than that of the CA3 (*DG, n= 9; CA3, n=8; ***P=0.0008*). **(E)** The reactivation rate of the DG when normalized to the chance level is still significantly higher than the chance level (*DG, n= 9; *P=0.0134*), whereas the reactivation rate of the CA3 is not higher than the chance level upon applying the same normalization (*CA3, n=6)*. Means ± SEM are shown in (A) to (E), compared with two-tailed, unpaired *t*-test. All formulas used in quantification are described in the materials and methods section.

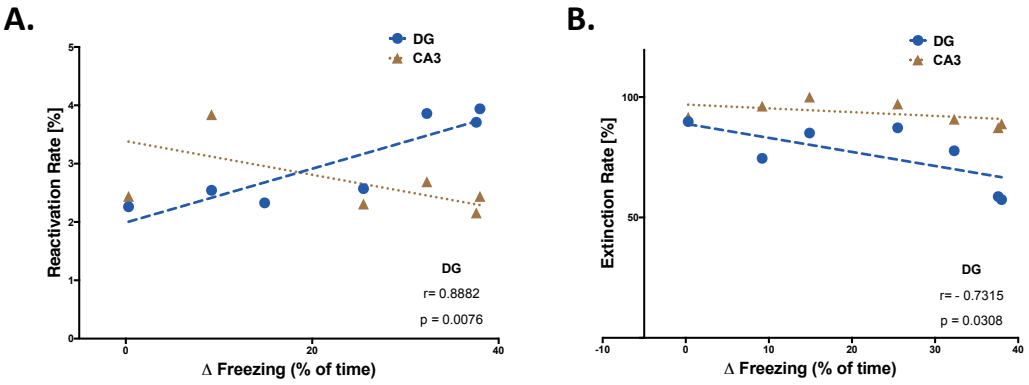

Figure 3.8: The reactivation of the recall-activated population during fear extinction provides a significant correlate for fear attenuation in the DG but not the CA3. (A) The reactivation rate in the DG (dashed blue line) is positively correlated with the Δ freezing (*DG, n= 7; r=0.8882, P=0.0076*), whereas in the CA3 (dotted brown line) it is not (*CA3, n= 6; r=-0.4683, P=0.3489*). **(B)** The extinction rate in the DG (dashed blue line) shows a weak but significant negative correlation with the Δ freezing (*DG, n= 7; r=-0.7315, P=0.0308*) whereas the extinction rate of the CA3 (dotted brown line) does not (*CA3, n= 7; r=-0.5437, P=0.1036*). DG: blue circles; CA3: brown triangles.

Additionally, the results I obtained through quantifying the immunolabelled cells were confirmed by cellular compartment analysis of temporal activity by fluorescence in situ hybridization (catFISH) [20,21]. In this experiment, another extinction

paradigm was used, the massed extinction, which has been shown to effectively attenuate remote fear [16]. Also another tool for detecting activated populations was used, the fluorescently labeled RNA probes performing fluorescence *in situ* hybridization (FISH). Briefly, wild-type animals were subjected to the same experimental design that was used and described before (Figure 3.3 A) with the exception of carrying out the massed extinction (c.f. materials and methods section) instead of the spaced. In catFISH, two fluorescently labeled RNA probes are used to detect IEG markers, namely, Homer1a (H1a), and cFos. Upon neuronal activation, IEGs are rapidly transcribed in the nucleus of these activated cells, and the cFos mRNA remains nuclear for 5 min before being transported to the cytoplasm. In contrast, H1a shows a delayed pattern of transcription such that it appears in the nucleus after 25-30 min post-activation, and can still be detected in the cytoplasm after being translocated for a period up to 70 min from the activation [20,21].

By using both probes, I managed to identify the activated cells during the recall (cytoplasmic H1a) and during the extinction (nuclear cFos) as represented in (Figure 3.9 A). Firstly, the behavioral readout of this experiment seemed consistent with the readout of the spaced extinction (Figure 3.3 B, and 3.4 D), where upon remote recall the animals showed prominent freezing that has been significantly reduced following massed extinction (Figure 3.9 B).

Furthermore, the activation rates quantified using catFISH in this experiment showed a similar pattern to the results of the previous experiment using immunolabelling. For example, the activation rate of both DG and CA3 showed high significance compared to their controls (Figure 3.9 C), whereas the learning rates of both regions were not significant from their controls (Figure 3.9 D). Finally, the reactivation rate at the DG was not only significantly higher than in the CA3 (Figure 3.9 E), but was also still significant when normalized to the chance level of activation, which was not the case for the CA3 that showed insignificant reactivation when compared to the chance level (Figure 3.9 F).

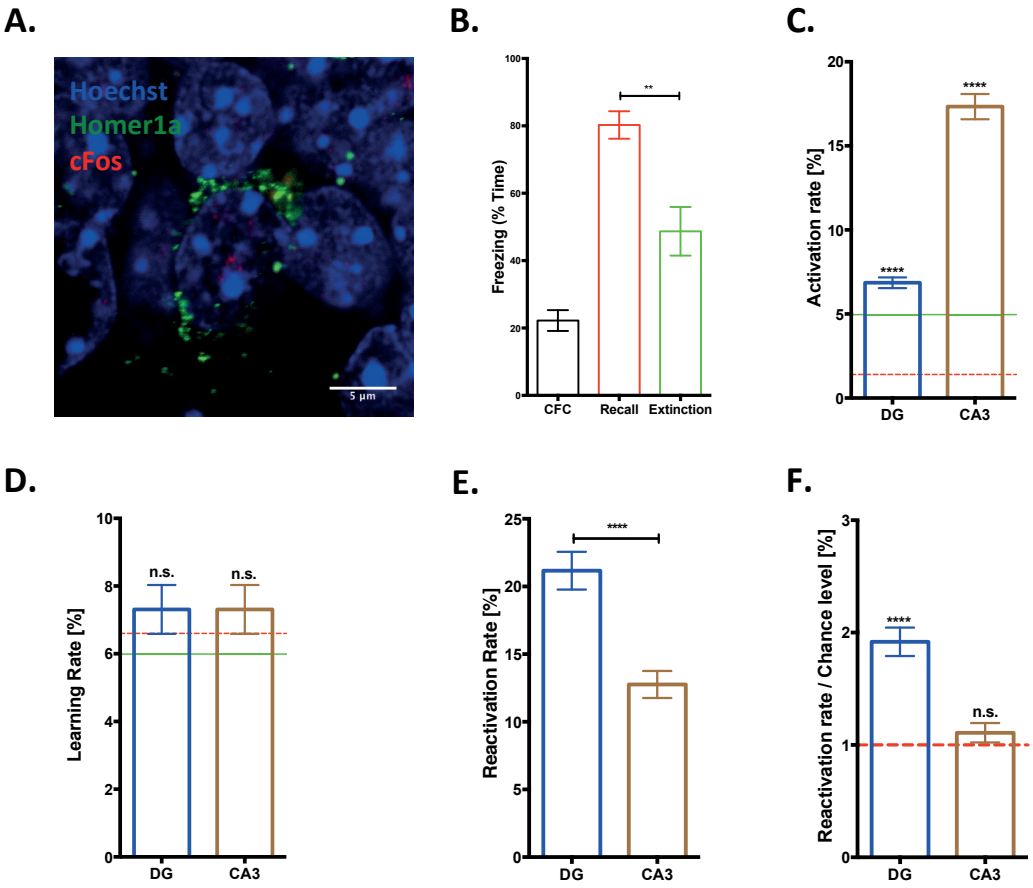

Figure 3.9: catFISH highlights the distinctly activated neuronal populations during the remote fear recall and extinction confirming the immunolabelling results. (A) Representative image of the catFISH experiments. Hoechst (in blue) stains the nuclei, whereas cytoplasmic Homer1a (in green) demonstrates the activation of this cell during the recall test, while the nuclear cFos (in red) indicates the activation of this cell during the last epoch of massed extinction. Scale bar, 5 µm. **(B)** The difference in freezing between the three epochs of training, recall, and last epoch of massed extinction shows a high freezing during the recall which can be significantly reduced after a massed extinction paradigm (**$P=0.0089$). **(C)** The activation rate of the DG and CA3 is significantly higher than the activation rates of the control groups (DG control group, red dashed line; CA3 control group, green solid line) (****$P<0.00001$). **(D)** The learning rate of the DG and CA3 is not significantly higher than the learning rates of the control groups (DG control group, red dashed line; CA3 control group, green solid line). **(E)** The reactivation rate of the DG is significantly higher than that of the CA3 (****$P<0.0001$). **(F)** The reactivation rate of the DG when normalized to the chance level is still significantly higher than the chance level (****$P<0.0001$), whereas the reactivation rate of the CA3 is not higher than the chance level upon applying the same normalization. [Animals used for massed extinction: $n=5$; control animals: $n=3$]. Means ± SEM are shown in (B) to (F), compared with two-tailed, unpaired *t*-test. All formulas used in quantification are described in the materials and methods section.

To recapitulate, the results of this section demonstrate that there is certainly a significant reactivation - above the mere chance - of the recall-induced population in the DG but not in the CA3 as demonstrated by both IHC and catFISH (Figure 3.7 E, and 3.9 F), and the majority of this population seem to be excitatory neurons verified by the overlap with CamKII (Figure 3.6). Finally, this significant reactivation of the recall-activated population provides a neural correlate for fear attenuation in the DG but not the CA3 (Figure 3.8).

3. Inactivating the reactivation of the recall-induced population in the HPC

After showing that there is a significant degree of reactivation to the recall-induced population upon fear memory extinction in the DG, I wondered if this significantly reactivated population is indeed essential in the process of extinction, and what will be the outcome following loss-of-function of this population. To this end, I planned the forthcoming experiments to address the second aim of my research question and to be able to verify the necessity of such reactivation towards remote fear attenuation. The following subsections are explaining how.

A. Validation of the Daun02-inactivation method *in vitro*

Before proceeding with inactivating the recall-activated population, the efficiency of the method I plan to use had to be tested. Despite the proven efficacy of the Daun02-inactivation method in previous research, I wished to show such effectiveness in primary cultured neurons. Briefly, the Daun02 is a prodrug that is completely inert, however it can be transformed into the active form Daunorubicin by the help of the enzymatic action of the β-galactosidase enzyme. This active form of Daun02 is known to reduce the Ca^{2+}-ion dependent action potentials in neuroblastoma cells, leading to the inactivation of those cells [22,23]. To test this, we designed a viral vector "DREADD-lacZ" (Figure 3.10 A) that can drive the expression of *lacZ* gene, as well as an excitatory synthetic receptor "hM3Dq" that is fused to an mCherry reporter gene. The hM3Dq is a designer receptor exclusively activated by designer drug (DREADD), and can only be activated if the synthetic ligand Clozapine N-Oxide (CNO) is found in the medium. The idea is to infect rat primary culture neurons with the construct expressing the hM3Dq, as well as the βgal enzyme. Upon adding the CNO to the medium, the cells expressing the hM3Dq will be strongly activated. Yet, if Daun02 is added with the CNO, then this activation will be abolished because of the transformation of the Daun02 into the active form Daunorubicin that will block any further activation to these neurons (Figure 3.10 B).

When the neurons were infected with the DREADD-LacZ construct, I observed that 30uM of CNO is indeed sufficient to cause a high surge of activation of the neurons as measured by an increase in cFos expression. But when 45nM of Daun02 is combined with the CNO, the level of cFos expression could not be increased, and it was significantly lower than that of the CNO alone condition (Figure 3.10 C and D). To control for this, some neurons were infected with another construct "DREADD-Zcal"that contains the hM3Dq, but contains the *lacZ* gene in the reverse frame rendering it defective. Upon adding the CNO alone, the cFos expression was increased as expected, and afterwards when the CNO and Daun02 were combined, there was no blocking to the expression of cFos (Figure 3.10 C and D) proving the effectiveness of the Daun02-inactivation strategy *in vitro*.

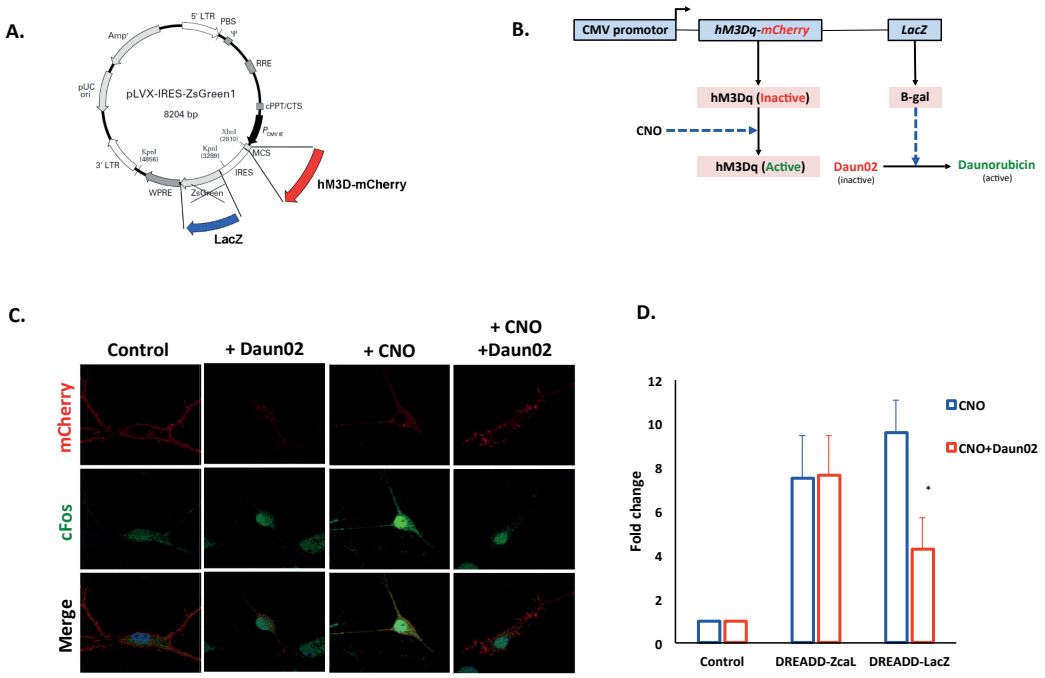

Figure 3.10: The Daun02-inactivation strategy confirms effectiveness in primary neuronal culture. (A) Design of the viral plasmid construct used to infect primary culture to express the excitatory DREADD (hM3Dq) with mCherry as a fusion protein, as well as expressing the *lacZ* gene. **(B)** Representation of the principle behind the Daun02-inactivation approach. Briefly, the infected primary neurons will be expressing both the hM3Dq and the *lacZ*. Upon adding CNO to the medium, the neurons expressing the hM3Dq will be activated and increase of cFos expression can be detected. However, if the Daun02 is added to the medium, the neurons expressing the *lacZ* gene will have the β-gal protein active and convert the Daun02 into Daunorubicin, which prevents any further activation of this cell by the CNO. **(C)** Representative images to the quantified cFos signal of **(D)**. In the cFos channel, the +CNO condition shows higher signal intensity, and upon combining the Daun02 with the CNO, this high signal intensity can no longer be attained as per quantified in (D). **(D)** Quantification of the signal intensity of cFos expression following an immunocytochemistry protocol. Infecting the

neurons with the DREADD-lacZ construct showed a significant reduction in activation if CNO (30uM) and Daun02 (45nM) were added together when compared to adding CNO alone (*$P<0.05$). There is no difference between both conditions upon infecting the primary neurons with the DREADD-Zcal construct, which contains the *lacZ* in the flipped reading frame. Means ± SEM are shown in (D), compared with two-tailed, unpaired *t*-test.

B. Examining the Daun02-inactivation method *in vivo*

Validating the inactivation method of Daun02 in primary culture neurons allowed me to proceed and test blocking the activation of the recall-induced population and observe the ensuing effect on the behavior after extinction training. To realize this goal, a cohort of TetTag animals was CFC trained as described previously, and two weeks later a bilateral cannula was implanted at the dorsal hippocampus to infuse the drug afterwards to the DG. The animals were left 10 more days to recuperate, and three days before the recall the DOX was removed from their diet to allow labelling the cells that will be active during the recall session. Ninety minutes following the recall, 2 ug of Daun02 or its Vehicle (VEH) was infused through the bilateral implanted cannula. The animals were then kept on DOX, and the following day the spaced extinction was conducted as previously described (c.f. Materials and Methods). Then the animals were sacrificed following the last extinction (Figure 3.11 A).

On the behavioral level, and during the recall session both groups showed prominent freezing indicating the successful retrieval of such remote fear. Also at the last session of extinction, both groups showed significant attenuation to the fear (Figure 3.11 B). Nevertheless, only the Daun02-injected group showed a significantly lower level of Δ freezing indicating a reduced degree of extinction when compared to the VEH-injected group that showed a significantly higher level of Δ freezing (Figure 3.11 C). Afterwards, the cannula traces were identified in the brains of both cohorts and the ones with deviations from the targeted region were excluded (Figure 3.11 D). Moreover, and to visually observe the Daun02-mediated inactivation, an X-gal staining protocol was performed. The main notion is that the X-gal staining is directly proportional with the amount of active β-gal molecules present in the cell. The more the cell is being activated the more copies of β-gal will be expressed, but if the cell is inactivated, then the β-gal expression will halt and this will be reflected by the

intensity of the X-gal staining. Thus, when the brains were processed for the X-gal staining, the Daun02-injected brains showed remarkably less staining compared to the VEH-injected group indicating the inactivation of the β-gal-expressing cells by the marked reduction of β-gal expression (Figure 3.11 E).

To confirm that the observed reduction in Δ freezing in the Daun02-injected group is indeed true and not an artifact, I performed several control experiments to rule out such doubt. The first control (CA3 group) proceeded with the same experimental design like in the previous experiment, yet the cannula was implanted to target the CA3 field (Figure 3.12 A) where the reactivation rate was not significant from chance level (Figure 3.8 A). Infusing Daun02 to the CA3 blocking the reactivation of the recall-induced population there did not have any effect on the extinction when compared to the VEH-injected group (Figure 3.12 B). The second control group (DOX ON Group) underwent the same experimental procedure as the experimental group, i.e. Daun02 was infused into the DG, yet the DOX-containing diet was never switched off, so that the recall session would not drive the expression of β-gal in the population that will be activated during the recall (Figure 3.12 C). The impact of Daun02 and VEH was comparable with no effect on the degree of extinction (Figure 3.12 D). Finally, a third control (WT group) was used to rule out that the Daun02 itself could affect the behavior of the animal. Therefore, the experimental design of the main experiment was repeated, including DOX removal, but this time with wild-type animals (Figure 3.12 E). Infusing the Daun02 in the DG of these animals did not affect their extinction capacity when compared to their counterparts that were injected with the vehicle (Figure 3.12 F).

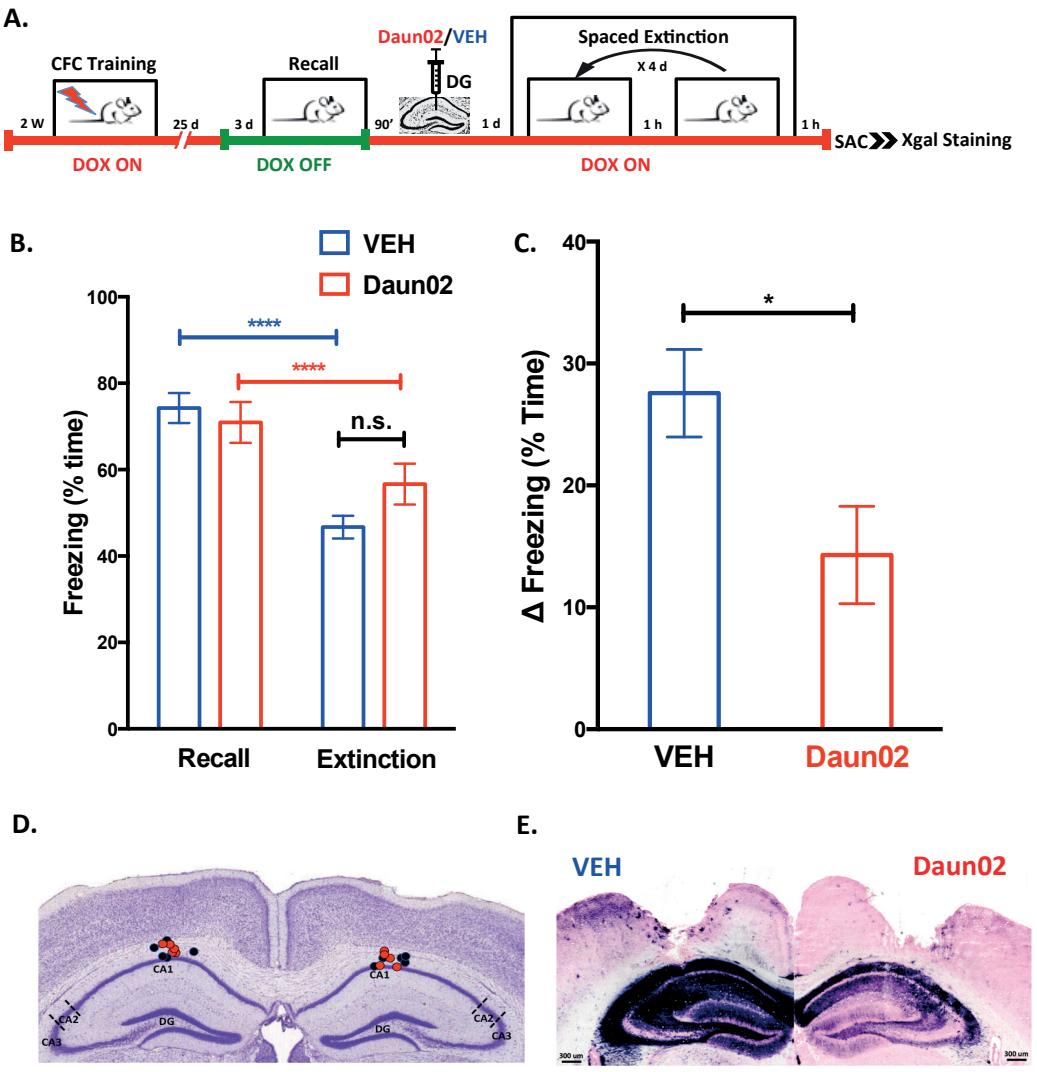

Figure 3.11: Using the Daun02-inactivation approach to block the reactivation of the recall-activated population affected the extinction of remote fear. (A) Timeline of experimental design. Briefly animals were contextually fear conditioned, and 90min after recall – while off DOX - the Daun02 was infused locally to the DG. The spaced session was performed the next day as usual, and the freezing behavior was recorded. The X-gal staining was performed after sacrifice to verify effectiveness of the used method. **(B)** Behavioral outcome of the experiment. Both groups show significant reduction of freezing following extinction (*Daun02, n=9; VEH, n=9; ****P<0.0001,* compared with two-way ANOVA: behavior session x group interaction, $F_{1,32} = 2.761$, $p=0.1063$; main behavior session effect, $F_{1,32} = 27.44$, $p<0.0001$; main group effect, $F_{1,32} = 0.6892$, $p=0.4126$). **(C)** Despite reaching significant attenuation of fear, the level of attenuation of the Daun02-injected group was significantly reduced when compared to the VEH-injected group as measured by Δ freezing (*Daun02, n=9; VEH, n=9; *P=0.0250*, compared with two-tailed, unpaired *t*-test). **(D)** Illustration of a mouse brain coronal section (adapted from Allen brain atlas) showing the stereotaxic sites of Daun02 infusion in the dorsal HPC to the DG. **(E)** Representative images of X-gal staining from the VEH-injected group (left) and the Daun02-injected group (right) showing a clear reduction in staining in the Daun02-injected group due to blocking by Daunorubicin (the active form of Daun02) and a subsequent reduction of β-gal expression. Scale bar, 300 µm. Means ± SEM are shown in (B) and (C). [CFC: contextual fear conditioning; SAC: sacrifice].

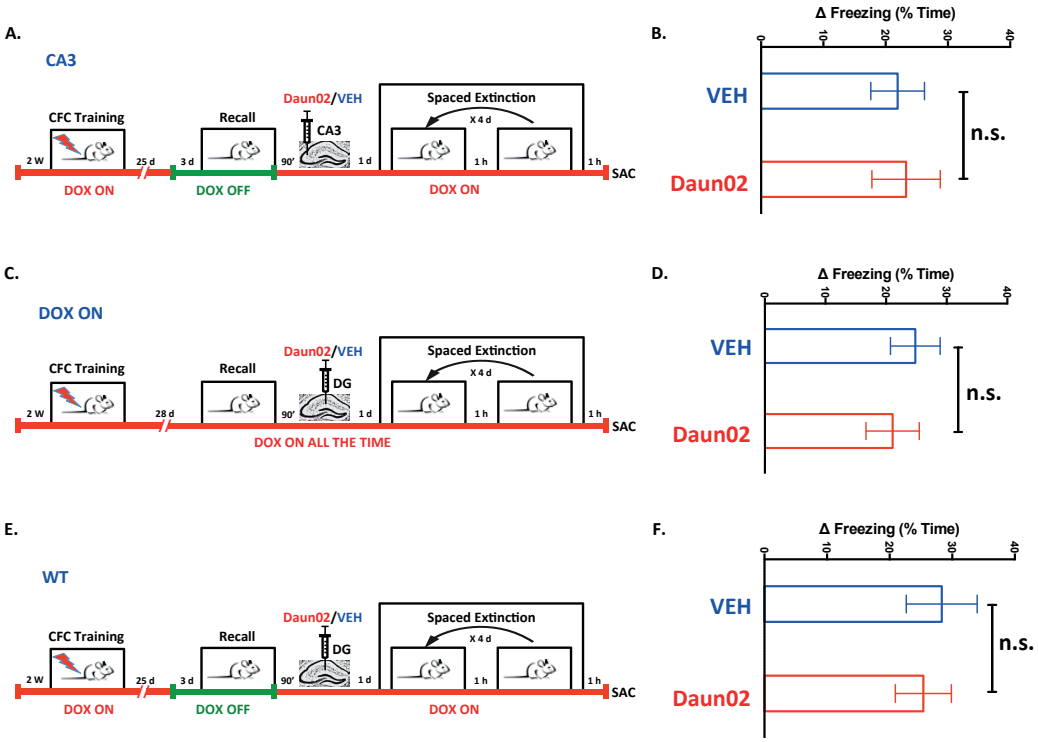

Figure 3.12: The Daun02-inactivation method does not affect remote fear extinction in the CA3 or the other control groups. (A, C, and E) Timelines of experimental design. Briefly animals were contextually fear conditioned, and 90min after recall the Daun02 was infused locally to the CA3 (A), or the DG while the DOX is ON the whole time (C), or in the DG using WT animals and not TetTag ones (E). The spaced session was performed the next day as usual, and the freezing behavior was recorded. **(B, D, and F) Δ** freezing of the different control experiments. There is no significant difference between the Daun02-injected cohort and the VEH-injected one in the CA3 groups (B) *(VEH, n= 9; Daun02, n= 8)*, neither in the DOX ON groups (D) *(VEH, n= 8; Daun02, n= 9)*, nor in the WT groups (F) *(VEH, n= 9; Daun02, n= 13)*. Means ± SEM are shown in (B) to (F), compared with two-tailed, unpaired *t*-test.

In summary, the results of this section demonstrate that the Daun02-inactivation method is surely effective in blocking further activation of neurons expressing β-gal as shown *in vitro* in primary culture (Figure 3.10). Furthermore, the efficiency of the Daun02 method has been also demonstrated *in vivo* (Figure 3.11) blocking the reactivation of the recall-activated population and affecting the overall attenuation of fear. These results speak in favor of the necessity of such population for successful fear attenuation, and that the loss-of-function of this population could reduce the competence of fear attenuating strategies.

4. Synthetic activation of the recall-induced population in the HPC

Showing that the reactivation of the recall-induced population in the DG is necessary during extinction for achieving effective attenuation of fear, made me wonder what would be the result of synthetically activating this population and not blocking it. To address the third aim and identify the influence of this population, I used a single transgenic mouse line (Fos-tTA) to train for CFC. Seven days later, and while on DOX-rich diet, I stereotaxically delivered an adeno-associated virus (AAV) vector to the DG of the animals. This viral vector drives the expression of the excitatory DREADD hM3Dq (fused to the fluorescent reporter mCherry) under the tetracycline-dependent promotor (TRE). Animals were then left three weeks to allow enough time for cells to be infected. Five days before the recall, the DOX was switched off of the diet, and the concept is that the recall session will drive the expression of the hM3Dq in the recall-induced population. Consequently, this population will be amenable to synthetic activation upon injecting the CNO systemically. The synthetic activation was carried out thirty minutes before the extinction session and repeated throughout the extinction days (Figure 3.13 A).

Evaluating the behavioral outcome of this manipulation showed that the synthetic activation of the recall-induced population indeed enhanced the degree of fear attenuation significantly when compared to the VEH-injected cohort (Figure 3.13 C). The animals' brains were processed to examine the hM3Dq expression and exclude the animals that had no expression or more diffused one in another region other than the DG (Figure 3.13 B). Nevertheless, I would still need the results of the currently running control experiments to be able to confirm that this outcome is indeed accurate and not just an artefact. The controls that are being currently carried out are reiterating the same controls from the Daun02-inactivation experiment (Figure 3.12), but this time I would like to check the effect of the synthetic activation of the recall-induced population at the CA3, or in the DG while the animals were never allowed to tag this population of cells with the excitatory DREADD (i.e. all the time on DOX), or what would be the effect of CNO on a wild-type cohort.

Finally, the results of this experiment add up to the findings of the previous experiment inactivating this population. It appears that the recall-induced population in the DG is quite central for the extinction treatment to work effectively. The pharmacological inactivation leading to loss-of-function of such population affected significantly the attenuation of fear (Figure 3.11 C), whereas the synthetic activation lead to a gain-of-function that enhanced attenuating fear (Figure 3.13 C).

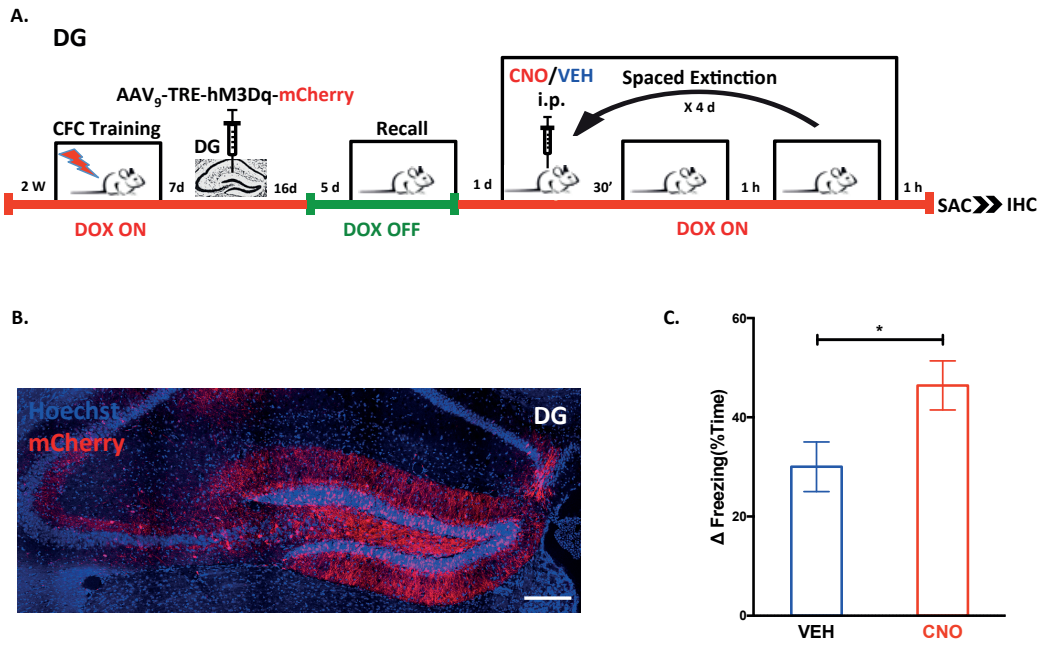

Figure 3.13: Synthetic activation of the recall-activated population improves the extinction of remote fear. (A) Timeline of experimental design. Briefly animals were contextually fear conditioned, and 1W later a viral vector expressing excitatory DREADD (hM3Dq) under a doxycycline-controlled promotor is locally delivered to the DG. 3W later, the recall session is performed off DOX, and 1d later the spaced extinction was conducted as previously mentioned in the materials and methods but an i.p. injection of CNO was given 30min before the extinction sessions. The freezing behavior was recorded to assess the degree of extinction. **(B)** A representative image of the expression pattern of hM3Dq in the DG. The nuclei are stained with Hoechst (blue), and the mCherry fusion protein (in red) shows where the DREADD receptor was expressed in the HPC during the remote fear recall. Scale bar, 200 µm. **(C)** The Δ freezing of the CNO-injected group shows a significant improvement in fear attenuation compared to the VEH-injected group (*VEH, n= 13; CNO, n= 16; *P=0.0290)*. Means ± SEM are shown in (C) compared with two-tailed, unpaired *t*-test.

5. Towards a molecular dissection of a reactivated population

Defining the population that is activated during the remote fear recall and get reactivated during the extinction allowed us to examine its influence on fear attenuation. However, and what comes in mind now is to ask what molecular processes are taking place in this rather small, yet unique population. To come closer to an answer for the fourth aim of my research project, I performed the following experiments:

A. Detecting epigenetic modification

Trying to unearth what makes a small population of cells that was once active recalling a remote fear get active again during its extinction, I decided to start with simple approach, i.e., to measure the acetylation of H3K9 which has been shown to increase facilitating memory extinction [24]. After performing the co-immunostaining (Figure 3.14 A), and assessing the intensity of AcH3K9 co-labelling with the reactivated cells in the HPC, I observed a significant increase in acetylation in the reactivated population (double+) when compared to the cells that were not active in either of the recall and extinction sessions (double -) (Figure 3.14 B). Such increase in acetylation for this histone mark could be one of the factors regulating the reactivation of this unique population.

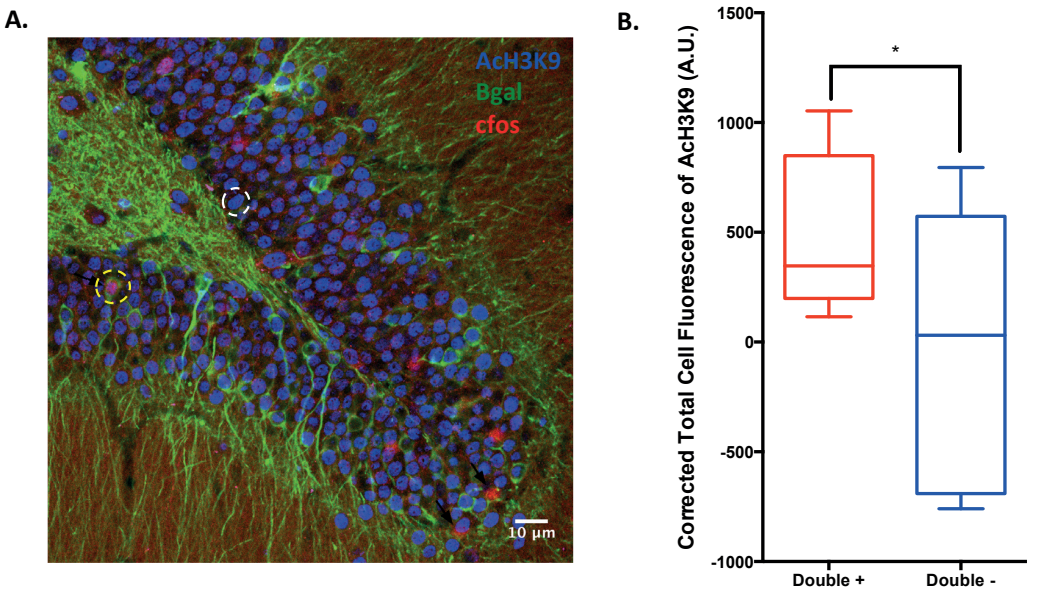

Figure 3.14: Reactivation of recall-induced population shows a significant increase in the acetylation of H3K9. (A) A representative image showing the co-labelling of the cFos/ βgal double positive cells to the AcH3K9. The intensity of the AcH3K9 signal was measured densitometrically. Scale bar, 10 µm. **(B)** The intensity of the AcH3K9 signal co-localizing with the cFos/βgal double positive cells is significantly higher than its counterpart of the double negative cells (*different animals analyzed n=2; cFos/βgal double labeled cells analyzed, n=10; double negative cells analyzed, n=13; *P=0.0431*). Means ± SEM are shown in (B), compared with two-tailed, unpaired *t*-test.

B. Cell sorting

One important molecular process to know about the reactivated population is the gene expression pattern in it. Knowing what genes are being expressed and how much copies are being made was amongst the main questions I wanted to address. Consequently, I thought of carrying out RNA sequencing on a single-cell level to be able to access the information of the entire transcriptome of the cells of this population. But first, one needs to physically isolate these cells to be able to analyze them later, and here I describe the approaches I used for cell dissociation and then for sorting the desired ones.

For the FACS experiments, I used the same experimental design as in the behavioral experiments (Figure 3.4 A) but the mouse line I used was expressing tdTomato

fluorescent protein instead of β-gal (Figure 3.15). In this mouse line, the cFos promotor not only drives the expression of the tTA transcription factor, but also the expression of a short half-lived version of GFP, namely: Enhanced GFP (EGFP). And since the main goal is to be able to extract and sequence the RNA from the collected cells, it was imperative not to fix the cells, and for that reason the aforementioned line comes handy. It provided a great solution to collect the cells that will be activated during the recall and extinction sessions without resorting to fixation. During the recall, the DOX-rich pellets will be off of the diet, and the recall-activated population will start expressing tdTomato. Afterwards, and during the extinction days, the animals will be kept on DOX-rich diet, and 1h following the last extinction session the animals were sacrificed and their brains were extracted and quickly dissecting out the HPC. Then, a cell dissociation protocol was carried out (as described in the materials and methods section) and the cells were taken directly to the cell-sorter.

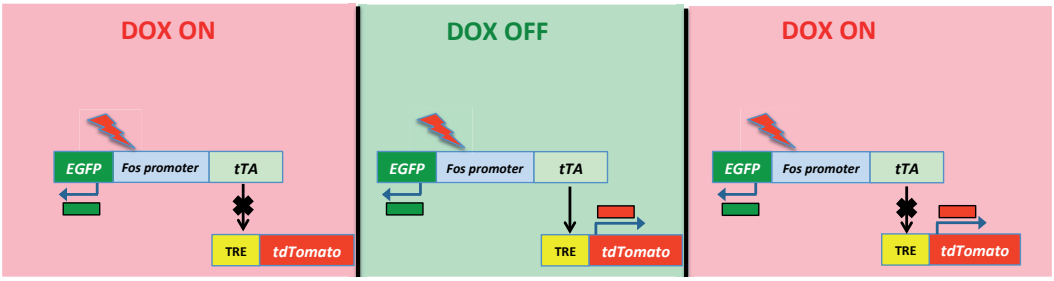

Figure 3.15: Another variant of the double transgenic TetTag system used for sorting. This line is similar to the classical TetTag, with the difference of the reported gene. In this line, the window of tagging can be opened by switching DOX off of the diet of the animal. The tTA will consequently be able to activate the tetracycline responsive element (TRE) to drive the expression of the fluorescent protein tdTomato (tdT). Another fluorescent protein (EGFP) is being expressed in this line upon activation of the cFos promotor and this gene is not controlled by DOX, yet it has very short half-life.

First steps of sorting is to be able to detect the debris from the intact cells, and this has been performed using the size of both and according to their side and forward scatter properties a gate was set to only include the cells for the next step (Figure 3.16 A). Then, and based on the height and width of the events (i.e. cells), a gate was set to only take into account single cells and not any two or more cells that are clumped together (Figure 3.16 B). Following comes the gate where the signal received from the nucleated (Hoechst+) and still alive i.e. propidium iodide negative (PI-) cells is being considered and passes to the following (Figure 3.16 C) and last gate. In the last

gate, only the cells that are single, nucleated (Hoechst+), and still alive (PI-) will be considered and their fluorescence will be checked to set up the final gates before sorting the cells. In these gates a threshold will be made to consider cells either double negative (DN), or tdTomato positive (tdT pos), or EGFP positive (EGFP pos), or finally double positive (DP) (Figure 3.16 D). In Figure 3.14 E, it shows the set thresholds for the 4 populations using a WT group as a control since they do not have these fluorescent proteins. In Figure 3.16 F, the sample has been sorted showing the cells that have been collected based on their fluorescing signals and which population they are part of.

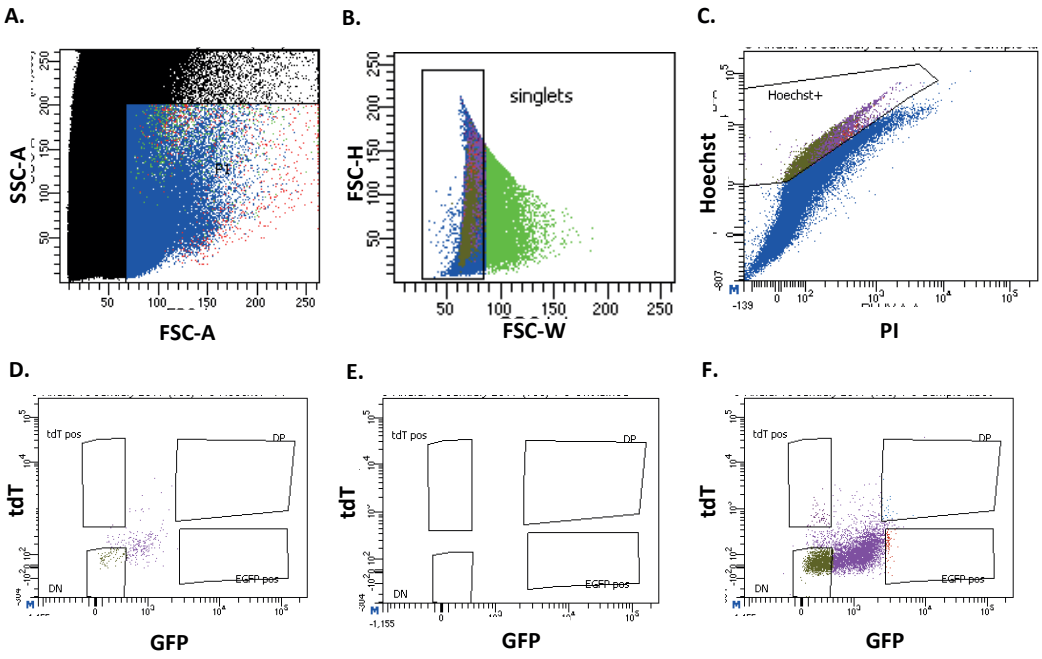

Figure 3.16: FACS Sorting plots showing the rigorous steps of sorting the recall-induced reactivated population. (A) A sorting plot of the forward and side scatters where the cells are being distinguished from the debris based on their physical properties (mainly dimensions, and surface area). **(B)** Sorting plot of singlets (single events/cells) excluding doublets (more than one single cell clumping to another). **(C)** A sorting plot showing the nucleated cells that are positive for Hoechst (upper region of the y-axis), and negative for the viability marker propidium iodide (left region of the x-axis). **(D)** A plot showing the gatings constructed using the Hoechst+ and PI- cells from (C). **(E)** Sorting plot of the control sample of a wild-type animal that does not contain any of the fluorescent proteins sought after. **(F)** Final sorting plot showing the 4 strict gatings used to collect the 4 populations based on the data generated from the sorting of the control in (E). DN (double negative, lower left quadrant); tdT pos (tdT positive, upper left quadrant); GFP (EGFP positive, lower right quadrant); DP (double positive, upper right quadrant).

Furthermore, the four populations were collected in 384-wells plates (single cell/well). The four populations were collected (96 cells of each population per plate) for the RNA sequencing and for sake of comparing the differential gene expression amongst the four populations. Finally, the quality of the RNA afterwards has been assessed using a fragment analyser to ensure the quality of RNA before proceeding to the sequencing (Figure 3.17). However, the results from the sequencing till this very moment of writing these lines have not yet been collected.

Prospectively, one expects that the sequencing data could show an important differential expression of certain genes in all four populations allowing us to identify some of them as target gene(s) that might be responsible for and/or regulating fear attenuation. Only then, such gene(s) would be themselves the target of translational medicine as they might be relevant for clinical research towards devising new approaches for fear attenuation.

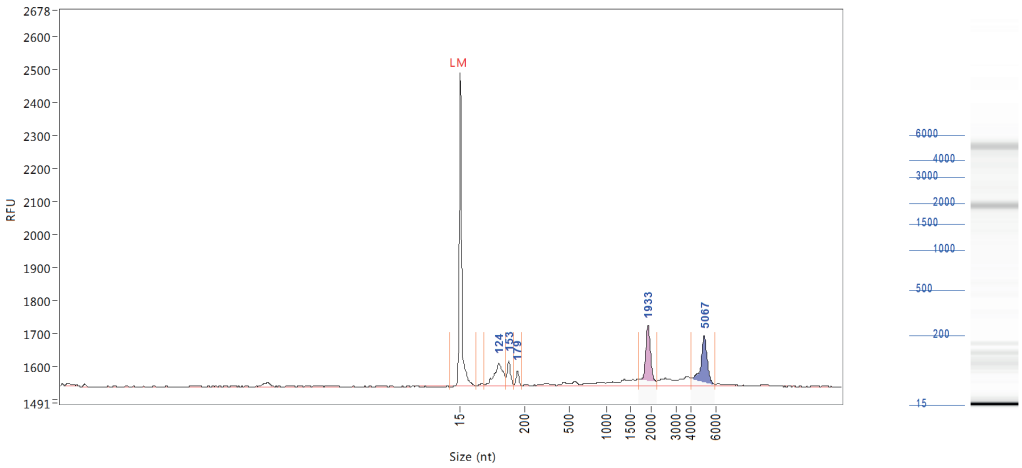

Figure 3.17: Quality control on the extracted RNA from the sorted cells. Using the fragment analyzer to ensure the quality of the extracted RNA from the cells before performing the sequencing. The agarose gel (on the right) shows a decent band sizes of both 28S and 18S ribosomal units. The migration times are shown (on the left) with the LM marker. The RQN is 6.9, which is reasonable to proceed for sequencing [98].

3.2 Discussion

Making sense of what we do!

In this study, I sought after identifying the neuronal subpopulations that are activated during successful memory extinction of remote fear, and to determine the molecular processes underlying such successful attenuation of fear. For that reason, I have embarked on using the double transgenic TetTag mouse line to address the specific aims of this project. But before delving deeper towards interpreting the results of my specific aims, I had to show first that the foreign genes that are inserted in this mouse line does not affect the behavior of these animals when compared with their wild-type counterparts. Performing an open field test which has been established as a method of assessing the anxiety of rodents [99], it showed that the TetTag animals do possess locomotive, exploratory, and overall behavioral traits no different than that of the wild-type controls (Figure 3.2). These results were vital to give me the green light to proceed with this line addressing my particular aims.

1. Reactivation of the recall-activated population provides a significant correlate for fear attenuation at the DG

Proceeding with the core experiment of the project, which is to identify the populations that are activated during recall and extinction, it was imperative to –first – show that the animals indeed learned fear as shown by the prominent freezing during the remote recall test (Figure 3.4 D). Processing the brains of the animals afterwards allowed me to visualize the cells activated during both epochs using immunostaining against β-gal as readout of recall-activated cells, and against cFos as readout of extinction-induced cells (Figure 3.5). It has been reported previously, however, that there is a problem of β-gal induction in the HPC [100] as well as in other brain regions like the basolateral amygdala [97], and due to this reason and the poor labeling I encountered in some of these regions as well, I decided to further investigate the regions that showed a clear, consistent signal, namely the DG and CA3. It was then expected to detect the nature of these activated neurons and in line with what has been shown by Tayler and colleagues, the nature of the activated neurons was almost entirely limited to excitatory neurons as shown by the CamKII co-immunostaining [101]. However, in my case I had trifling amount of cells that were being co-labeled with GAD67 the inhibitory neuron marker, but this might be attributed to the cells that were activated unspecifically due to the basal activity of the animals while the DOX-rich diet is off and not due to the specific activation due to the recall of the remote fear (Figure 3.6).

Next, the quantifications I carried out determining the amounts of cells activated during the recall showed that the DG and CA3 are significantly activated (12-13%) much more than the unspecific activation of the control groups (Figure 3.7 A). This was confirmed by catFISH that showed a similar readout of significant activation of both regions (Figure 3.9 C). Despite using different paradigms than mine, this result is similar also to what has been previously shown, namely that the HPC is highly engaged in remote recall [94,101–105].

Moreover, I wanted to check how these activation rates relate to what has been reported before especially those who have used the same labeling system i.e. the TetTag system. In Tonegawa's group, the activation rates described for the DG were almost half of my activation rate [106,107], and this might be due to using a viral approach to infect the DG with the TRE construct to control the expression of the reporter gene as compared to having it endogenously in the double transgenic animals I used (Figure 3.1). Another valid reason for this could be that they used the TetTag system to label the cells during the encoding of a context [107], or contextual fear [106], whereas I did to tag the cells responsible for the recall of fear. In another study by Tayler and colleagues using a similar double transgenic line like the one I used, the reported activation rate was similar to mine only at the CA3, but not the DG [101]. This might be for the very same reason mentioned before where the activation rate was quantified for the cells activated during fear conditioning and not its recall. Despite sharing the same tagging technique, the number of activated cells might vary between brain regions and due to using ever so slightly different behavioral tasks as in the previously mentioned references, and also in many others [97,100,108].

Next, I analyzed the cells that were activated during the extinction alone i.e. learning rate. It turned out that they were not significantly higher than those activated due to unspecific activity in the home cage or the context as shown by the number of cFos positive cells (ca. 2%) (Figure 3.7 D), and this was confirmed by the catFISH showing similar result (Figure 3.9 D). This might essentially be because the absolute number of activated cells is not what counts, and that the identity of these cells is what matters, as it has been shown by cellular studies of immediate genes that the pattern of activation is what changes in different environments [109] or due to different tasks [110] and not the absolute number of cells especially that the number of neurons recruited in learning at these regions – especially the DG - has been reported to be quite low (ca. 2-4%) [111].

It is noteworthy, however, that almost 25% of the cells that were activated exclusively in the extinction in the DG used to be active during the recall itself, while at the CA3 this percent was meager (Figure 3.7 C). Likewise, there was much more cells at the DG that were active in the recall and then reactivated in the extinction when compared to the CA3 (ca. 3%, and 1.5%, respectively) (Figure 3.7 D). The results of catFISH also confirm such finding of having significantly higher amount of cells at

the DG that get reactivated compared to those of the CA3 (Figure 3.9 E). Interestingly, only the reactivation at the DG was significantly higher from the mere chance of reactivation, whereas this was not true at the CA3 (Figure 3.7 E, and 3.9 F). This is in line with a similar mouse line expressing H2B-GFP instead of β-gal in which the overlap for a recent memory (2d) is quite significant in the DG but not the CA3, whereas for a remote memory (14d) it is not. Comparably, this is the case for the reactivation I obtained since it was between the recall and the extinction that are 4d apart rendering them a recent-like memory.

Having a significant reactivation in the DG made me wonder if this is indeed meaningful. Hence, and inspired by previous studies showing stable behavioral correlates [97,100], I plotted the individual reactivation rates of the animals against the Δ freezing. A significant positive correlation was observed suggesting that this reactivation might be essential for the animals to attenuate their fears. It was also expected that this would not be the case in the CA3 since the reactivation rate there was not significant from the chance level from the start (Figure 3.8 A). Expectedly, and by plotting the extinction rate and the Δ freezing, the DG showed a significant negative correlation whereas the CA3 did not (Figure 3.8 B). This latter correlation indicate that the fewer the cells that were exclusively active during the extinction the better the extinction will be since the rest of the cells that are activated during the extinction were activated during the recall once before.

Conclusively, these findings suggest that the significant reactivation of the recall-induced population in the DG provides a significant correlate for remote fear attenuation especially that it has been recently reported - using the very same mouse line – that neither home cage activity, nor that at the context alone has any effect on the reactivation pattern at the DG [100] positing that the significant correlation I have is quite relevant for fear attenuation.

2. Inactivation of the reactivated population in the DG impairs remote fear attenuation

After having found this significant reactivation in the DG from the previous experiment, and after showing that this reactivation suggests a stable correlate for fear attenuation, it was tempting to test the loss-of-function of such reactivation in the whole process of fear extinction. For this purpose, I decided to use the Daun02-inactivation method to block the recall-induced population from being reactivated during extinction and observe the effect on the attenuation of fear. Despite being used effectively to inactivate β-gal-expressing cells in several previous studies both *in vitro* and *in vivo* [27-31], I wanted to start by evaluating the efficacy of such method in my hands *in vitro* before proceeding to the *in vivo* study. The results from the primary neuron cultures were very promising, as the Daun02 is indeed mediating an inactivation to the β-gal-expressing cells (the neurons that were infected with a viral vector driving the expression of the *lacZ* gene) (Figure 3.10 C). This effect would essentially be due to the β-gal-aided enzymatic transformation of the Daun02, which is an inert prodrug, to the active molecule Daunorubicin, which in turn inactivates these cells through either of two potential mechanisms: apoptotic cell death [117,118], or blockade of Ca^{2+}-dependent action potentials [119] (Figure 3.10 B).

The *in vivo* experiment was performed following the successful demonstration of the efficacy of such technique on primary culture. The inactivation of the recall-activated population indeed affected the quality of fear extinction significantly as shown by the reduction in Δ freezing of the Daun02-infused animals when compared with that of the VEH-infused ones (Figure 3.11 C). The visualization of such inactivation using the X-gal staining was convincing (Figure 3.11 E), yet awaiting further proof that the behavioral effect is exclusively due to the reactivation block by the Daun02. The control experiments were thus critical to show that the Daun02-mediated inactivation in the CA3, or in the DG without removing the DOX from the diet (never opening the labeling window), or finally in wild-type control animals could not elicit any effect on the Δ freezing when compared to the VEH-injected controls (Figure 3.12 A-F).

The outcome of this experiment showed that not only is the reactivation of the recall-induced population in the DG significant and correlates with the quality of fear

attenuation, but also that it seems necessary for the attenuation of fear. One control experiment will be carried out soon – nonetheless – which is to inhibit some random cells at the DG and verify that it is not the amount of inhibited cells what matters, but what do they represent does. Such control has been reported previously in the memory allocation literature and others as well [104,120–123]. This would be essentially done by switching the DOX off of the diet of the animal and labeling the cells active in another context or even the home cage, and having the DOX on during the recall session – to keep the DG activated cells not tagged with β-gal – and afterwards infuse the Daun02 into the DG and observe the effect of inactivating this random population in the DG on the fear attenuation.

What is yet to be addressed is two main points: firstly, what if Daunorubicin inactivates the cells by ablating them through apoptosis, then some collateral effects could be imposed on the nearby neurons. This is rather unlikely since the DG neurons are in the vicinity of the CA3 region, and the CA3 control group would have displayed a similar impairment of fear attenuation as a result of an off-target apoptosis to the neighboring region i.e. the DG neurons. Moreover, it has been reported through the controls of other groups working on neuronal ensembles of drug addiction that such effect is improbable [112,113,124]. The second point is why not using an optogenetic silencing of this population, especially with its proven efficacy [103,104,106,107]? Amongst the advantages of optogenetic manipulation is that it lasts for a very short, which might not be enough for our purpose. For that reason, there is a control experiment I am currently conducting to see the effect of a proven reversible inactivation of such population using the inhibitory DREADD system. Briefly, I use a version of the TetTag that will express the inhibitory DREADD (hM4Di) during the recall i.e. when DOX is off of the diet. Then through a cannula implanted on the HPC, CNO will be locally infused to the DG, which will silence the recall-induced population for few hours. The goal is to check whether this temporary, and reversible silencing strategy will reproduce the behavioral phenotype I observed with the Daun02-inactivation approach.

Nevertheless, the optogenetic silencing approach is still in mind, and would be highly informative, especially that it has been reported that the precise – but not prolonged - inhibition of the CA1 impaired remote contextual fear recall [103]. Thus, I am curious to reveal its effect on fear attenuation by exclusively limiting such silencing to the 3-

min periods of extinction. Like that we will be able to better understand if the reactivation of the recall-induced population is exclusively necessary in real-time during the extinction sessions or is it imperative for such population to be active during these sessions and stay activated for a longer period afterwards. Only the pharmacological inhibition vs. the optogenetic one will be able to tell us.

3. Activation of the reactivated population in the DG boosts remote fear attenuation

Illusionists usually say that the effect of making a bird disappear is great, but to bring it back, the impact will be even bigger. Likewise, I felt tempted not only to block the reactivation of the recall-induced population like I showed in my previous experiment, but also to synthetically activate it to examine its gain-of-function on fear attenuation. To serve my purpose, I had a virus designed to deliver an excitatory DREADD (hM3Dq) to the DG of cFos-tTA animals, so as when the DOX-rich diet is switched off during the recall, the recall-induced population will this time express the hM3Dq that can later on be synthetically activated by the otherwise inert small molecule CNO (Figure 3.13). After verifying that the delivery and expression level of the DREADD is satisfactory (Figure 3.13 B), I observed that the synthetic activation of the recall-induced population is indubitably improving the fear attenuation as measured by the significant increase in Δ freezing of the CNO-injected animals compared to their VEH-injected counterparts (Figure 3.13 C).

Taking into account all results so far, the data would then suggest that this significantly reactivated population is necessary and influential for attenuating remote fear memory. It is noteworthy, that the control experiments for this experiment are currently being carried out. They essentially resemble the ones performed to confirm the authenticity of the Daun02 results. Also checking the gain-of-function of this population through the synthetic activation of optogenetic manipulation is something in mind and soon will be done. Another scenario I would check using the optogenetic activation is to examine if the whole sessions of extinction could be replaced by some bouts of synthetic activation in the home cage, and observe the effect on fear attenuation.

4. Increase in the acetylation of H3K9 could be contributing to the influence of the reactivated population on remote fear attenuation

The acetylation of H3K9 has been previously reported to facilitate fear memory extinction [125,126]. Hence, I thought of checking the AcH3K9 mark in the reactivated population. As expected the acetylation level was higher in the reactivated population when compared to the population that was not active neither during the recall nor the extinction (Figure 3.14 B). Such increase in acetylation for this histone mark could be one contributing factor – but not the only – regulating the reactivation of this unique population. This result is in line with what was reported about the increase in acetylation of H3K9 following remote fear attenuation [94]. One important experiment to perform -however – would be to prevent such increase of acetylation through impairing the nitrosylation signalling of histone deacetylases. If the acetylation of H3K9 is one of the regulating factors for the reactivated population to influence fear attenuation, then such impairment could confirm if this is true. As it has been shown before that reduced hippocampal nitrosylation signaling toward HDAC2 might prevent remote memory attenuation [94].

What is currently being performed and will impart fecundity of information - towards this aim - is the analysis for the transcriptome of this reactivated population. First, we will learn what genes are being expressed uniquely (or maybe in excess) in such population compared to the other populations that were not reactivated. Then, going deeper into checking what kind of epigenetic regulation that is taking place in the promotor regions of these differentially expressed genes. Once known, this will be a significant finding since it will provide us with information about the molecular signature - for the very first time - of a specific engram that is essential for fear attenuation. This could ultimately help us identify certain genes as targets for translational medicine to devise new strategies for fear attenuation and other anxiety disorders.

5. Conclusion, caveats, and beyond

The difference between "try" and "triumph" is just a little "umph"!

- Marvin Phillips

I started this project trying to identify the neuronal subpopulation that gets activated during remote fear extinction, and what could be its influence on the attenuation of fear. Designing the experiments, performing them, and afterwards analysing the results was incredibly thrilling for me, especially upon collecting the small pieces of the puzzle together to be able to deduce the bigger picture. But now, it is necessary to divulge what the results managed to answer so far, and what are the challenges and limitations that kept some issues not yet fully addressed, and could be improved through more precise experiments.

Firstly, the data inform us that there is a subpopulation of neurons in the DG that is activated retrieving the remote traumatic experience and then gets reactivated during the exposure-based therapy-like paradigm that aims at attenuating the fear accompanying recalling this trauma. This data suggest that the reactivation of such subpopulation is quite essential for the degree of the fear attenuation not to be compromised. The pharmacological activation of such population could even enhance the attenuation of fear if done during the exposure-based therapy sessions.

Secondly, so far it has been only shown through chemical lesioning or pharmacological inactivation that the DG is important for contextual fear encoding [127], or could impact spatial memory encoding but not its retrieval [128]. Optogenetic silencing of DG also had similar effects on encoding contextual fear without affecting its retrieval [129]. However, more precise chemogenetic or optogenetic inhibition for as little as 6-9% of DG cells that were previously active during learning impaired both memory encoding and retrieval [104,123]. Therefore, the results of my research are quite novel, since it has never been reported before that the inactivation of certain population in the DG that used to be active during the retrieval of remote fear is quite essential for this fear to be attenuated by exposure-based therapy. What has been shown before, however, is just the importance of

performing the recall and how it is essential for opening the time-limited window of reconsolidation for memory to be updated [43,94,102,130,131]. The novelty here is in allowing the recall to take place without real-time impairment of the entire process of retrieval, the only intervention comes afterwards when the cells active during the retrieval are tagged. These tagged cells are then, exclusively, manipulated and the rest of the DG is spared.

One caveat in my experimental design, however, is that I do inactivate a subpopulation of the DG and not its entirety, yet still constituting the entire population that was active during the recall i.e. ca. 12% of the DG, and not only the portion that gets reactivated afterwards i.e. ca. 3% of the recall-induced population. Given what I have discussed earlier about the more precise inhibition, and that the number of cells and length of inhibition amongst the factors that matter the most, I would expect that a similar phenotype -like the one I report here- might be achieved by targeting this smaller – yet more precise – population. Practically, I would need to have 2 independent tagging systems present in my animals – as if inserting 2 distinctive switches - to be able to label all the cells that will be induced by the recall session with one system, and then only the subpopulation of these activated ones that will be reactivated should have the second independent switch that will allow me afterwards to manipulate them separately from the rest of the cells. For this purpose, I suggest to combine the current intersectional strategies that use IEG-based models like recently shown by Yokose and colleagues [132].

Briefly, I would have bitransgenic mice that have (c-Fos::tTA/R26R::H2B-mCherry) and through lentivirus I would deliver (TRE::CreERT2, E-SARE::DIO-ArchT-EYFP). Like this I will have the c-fos-tTA TetTag switch [97] and lentivirus-based genetic targeting system in combination with a tamoxifen-inducible Cre-loxP recombination system [133] that will allow me to specifically target the overlapping ensemble. The procedure would be as follows: In a first step, the recall-induced ensemble will be labelled with CreERT2 under DOX off conditions. In a second step, DIO-ArchT-EYFP under the control of the enhanced synaptic activity responsive element (E-SARE) promoter [134] will be transcribed in the cells activated during the recall. Lastly, ArchT-EYFP protein will be translated only in those cells that are activated twice i.e. once during the recall and again during the extinction (the reactivated population). Like this, all the recall-induced population will have H2B-

mCherry, and only the reactivated subpopulation will have H2B-mCherry, and ArchT-EYFP. And by using optogenetics, it will allow me to specifically inhibit the overlapping smaller population and verify whether their loss-of-function will impair fear attenuation as well.

Thirdly, my results display that to attain a successful attenuation for remote fear the overlap between the population activated during recall i.e. the remote fear trace, and the one activated during extinction i.e. the reassurance/safe trace is crucial. This implies that fear attenuation does not result from having a completely "new" safe trace, nor the deletion of the "old" fearful one. It means that both traces are still there, yet the interaction of both – even if it is a very small overlap – is quite important for the new information of reassurance to be linked to the "old" trace of fear and consequently the animal relearns that the context it used to fear is now safe i.e. updating the old information that the context is harmful. Visualizing this essential overlap *per se* speaks in favor of an updated version of the original trace via reconsolidation as opposed to parallel traces of consolidation i.e. the fear trace is stabilized after recall, and the formation and consolidation of a completely different and parallel safe trace i.e. new learning leading to a new trace of extinction. The dissociation between both has been challenging since both consolidation and reconsolidation are sensitive to the same amnestic treatments aiming at distinguishing both processes [135]. Moreover, the results are quite consistent and in accordance with my understanding of how extinction works as per reported before for fear memories [130,131] and drug-induced addiction [136,137].

Prospectively, and besides the proposed optogenetic experiments for inhibiting and activating the reactivated population at the DG, it would be very informative to study how this population functions over time. This can be achieved using two-photon calcium imaging, where this population should be tagged with calcium indicators to be able to monitor the activity of such population *in vivo* over time similar to the approach that has been reported recently monitoring the participation of hippocampal granule cells to the encoding of novel information [138]. What could also be very enlightening is to make use of the recent microscopy and immunolabelling techniques that could allow us to visualize the activated populations across the entire brain using CLARITY [139], or PACT [140], or iDISCO [141], or CUBIC [142], or SWITCH [143], or any other similar technique that helps clearing the brain without affecting its

structural integrity. Also what could be very useful in the future to use is the recently announced product of iNSCOPIX that it is possible to combine calcium-imaging and optogenetics into the same implant (e.g. nVoke^{TM}), where it allows the monitoring and manipulation of tagged neurons *in vivo* [144].

Pertaining to limitations, an important one I would like to shed light upon is the TetTag window of tagging activated neurons. During my experiments, I had to make sure that the DOX is completely out of the animals' system before performing the behavioral test. This period used to be 3 days ahead of the test, which had to be controlled for using the other controls I previously discussed – and did use. The use of another control system like the Targeted Recombination in Active Populations (TRAP) line [145] would have provided a better resolution and a narrower time-window and the unspecific labelling would have been negligible.

Amongst other mundane challenges that deserve a notable mention:

1. The visualization itself of the activated population(s) required dealing with a transgenic mouse line the behaviour of which had to first be verified lest the transgenes are affecting it.
2. The behavioral readout of the animals – in terms of freezing percentage – was sometimes incongruent due to individual variations, or cage composition. Such disparity obliged me to have bigger cohorts and more animals in my experiments than anticipated.
3. The immunolabelling was complex especially that I had to try a "smorgasbord" of antibodies to settle on those that worked in my hands and in the brain region of interest.
4. Due to mediocre immunolabelling in other regions, I was obliged to stick principally to the HPC, and delay working on other equally important regions later, or as soon as I get better antibodies that will work well for these regions.
5. The cannula implantations, as well as the stereotaxic viral delivery proved to be quite demanding for the precision and meticulousness both require. Verifying the cannula trace and the viral transgene expression was key to confirm which animals to be taken into account and which will not be.
6. Finally, and the biggest challenge of all was the HPC dissociation into individual cells for sorting afterwards. I have tried countless number of

protocols that use different methods of dissociation and/or fixation, and/or staining. It was in sometimes frustrating that it does not work at all, or even when the sorting works but the RNA quality was not good enough for downstream processing.

Lastly, people thought flying or going to the moon is impossible, but with industry, perseverance, and unleashed imagination, it is just a matter of time to achieve anything one aspires to attain. I have, genuinely, encountered many challenges throughout my doctorate years, but they made my effort worthwhile, and the few "*eureka*" moments I had truly count. These defies kept my interest piqued all the way driving sleep to elude me sometimes. Without them, it would not have been the same. It would have been rather bland, and for all that I am quite appreciative!

Final Note:

Due to time constraint, and restrict submission deadline, I could not append a final section that instils my perspective about the field and how my data fit -if any. This section is nothing but a step back from the pixelated close-up, just to behold and appreciate the big picture. Nevertheless, this section will be ready to be discussed by the time of my *viva voce*, and eventually for the final version of the dissertation.

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Tribute to the giants we rest on their shoulders

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Appendix

Recent insights into remote memories

This section presents the following published review article:

Structural, Synaptic and Epigenetic Dynamics of Enduring Memories

O. Khalaf and J. Gräff – Neural Plasticity, volume 2016 - p. 3425908, 2016.

ABSTRACT

Our memories are the records of the experiences we gain on our everyday life. Over time, they slowly transform from an initially unstable state into a long-lasting form. Many studies have been investigating from different aspects how a memory could persist for sometimes up to decades. In this review, we highlight three of the greatly addressed mechanisms that play a central role for a given memory to endure: The allocation of the memory to a given neuronal population and what brain areas are recruited for its storage; the structural changes that underlie memory persistence; and finally the epigenetic control of gene expression that might regulate and support memory perseverance. Examining such key properties of a memory is essential towards a finer understanding of its capacity to last.

INTRODUCTION

Based on experience, memory is the capacity of an individual to acquire, store, and retrieve information. The physical substrate of such memories in our brains are known as memory trace or, as first coined by the German biologist Richard Semon (1859-1918), as "engram" [1–3]. One of the fundamental questions in memory research is how the experiences that we acquire transform into engrams that persist over time. It is generally acknowledged that the records we form from our daily experiences are not stored instantaneously, but rather retained in an initially labile state that gradually transforms into a more stable trace or engram that is characterized by resistance to disruption [4-6]. Although this view has been challenged by the reconsolidation hypothesis, stipulating that even a stably stored memory could become transiently sensitive to disruption upon recall [6,7], it is evident that not all forms of memories are amenable to disruption [8]. This is particularly relevant for strong memories, induced by an intensive training protocol, and long-lasting forms of memories, ranging from several weeks to months [9,10] in age. Based on these grounds, but notwithstanding several studies testifying to the amenability of even long-term memories to disruption [11,12], in this review we focus on 7d old – and older – memories as remote and with the potential to endure, and we outline three mechanisms that might contribute to such endurance: First, memory allocation and storage; second, structural neuronal changes; and third, nuclear epigenetic dynamics (Figure 1).

Memory allocation refers to an early process by which certain neural circuits are assigned to stow a specific memory, and what might favor the allocation of a memory into a specific population of neurons over others. In this review, we focus on some of the well-described elements that govern such allocation, still it is clear that we are only at the beginning of understanding the entire process of memory allocation, and many more aspects thereof remain to be identified. Once allocated, the question of where the memory is stored and what brain regions upkeep the memory is another one of utmost importance. The whereabouts of a specific memory is thought to be dependent on how old this memory is. The more nascent it is, the more it will be hippocampal dependent, but as it matures it will change such dependence to higher

cortical regions [13,14]. Here, we describe brain areas that have been defined to be essential for the support of a long-lasting memory.

Furthermore, many neuroscientists believe that memories are encoded into neurons as structural changes in synaptic connections. Indeed, such structural plasticity is under comprehensive study in order to understand how brain circuits are modifying themselves in terms of number and strength of synaptic connections that correlate with the persistence of a memory [15–17]. We discuss these physical changes in synapses and their potential to support enduring memories.

Lastly, we also discuss the epigenetic modifications that are associated with long-lasting memories. We shed light on such modifications to the DNA or the histone tails that could lead to a cascade of changes in gene expression – a key feature of long-term memories [18], and which might thereby be able to assist memories to persist throughout the life of an individual.

I. Memory Allocation and Storage

Once formed, memories gradually transform from an initially vulnerable state to a more permanent state that is increasingly persistent to disruption. Such process of post-experience memory stabilization was first described by Müller and Pilzecker referring to it as “memory consolidation” [4,5]. Later, two different types of memory consolidation have been distinguished; cellular/synaptic and systems consolidations. Cellular consolidation is a rather fast process taking place within the first few hours following learning and necessary for the initial stabilization of memories in hippocampal circuits [13]. In contrast, the systems consolidation process is slower and involves a time-dependent, gradual reorganization of the brain regions that support the memory, with the memory dependence shifting from the hippocampus to cortical regions [14]. This led to the contemporary view of systems consolidation which states that the hippocampus is merely a temporary store for new information, while its permanent storage depends on largely distributed cortical networks [14].

In this section, we review what molecular and cellular events govern memory allocation in or to a certain neuronal population and then, what brain regions support long-lasting memory storage.

A. Memory Allocation

By definition, memory allocation is the set of processes that determine where information is stored in a particular neural circuit [19]. Several studies showed that such allocation is not random, but rather dependent on specific molecular mechanisms [20–22]. In one of these studies[20], using a viral vector Han et al. artificially increased the levels of CREB (cAMP responsive element-binding protein), a transcription factor important for the stability of synaptic potentiation and memory [23] in neurons of the lateral amygdala (LA), a subcortical brain structure implicated in emotional memories [24,25], in mice. Twenty-four hours after a tone fear conditioning training, the mice were tested for the tone and sacrificed 5 min later. Using cellular compartment analysis of temporal activity by fluorescence in situ hybridization (catFISH), LA neurons transfected with CREB – identified by its GFP fluorescent tag – were found to be three times more likely than their neighboring non-transfected cells to express activity-regulated cytoskeletal (Arc), a gene required for synaptic function and memory [26,27]. This suggests that CREB levels bias neurons to become part of the engram and to be encoded by the tone conditioning in the amygdala.

In a subsequent loss-of-function study, cells that were virally transfected with CREB in the same behavioral paradigm were ablated using diphtheria toxin receptor (DTR). In this system, the expression of the DTR is inducible by the Cre-recombinase, which is also found in the same viral construct, making all the cells that receive the construct eventually express the DTR. Following the tone test (24h after training), the mice were injected with the diphtheria toxin (DT) that will only interact with the cells expressing the DTR and kill them. The experimental group (CREB viral vector transfected and DT injected) showed a significant impairment in tone conditioning when tested 2 days after the DT injection [21]. Similar results were obtained using a different approach that allows for reversible neuronal activation instead of

permanently killing the cells [22]. There, the Drosophila allostatin inhibitory receptor was delivered to the LA through the same viral construct providing CREB, and a pronounced amnesia for tone conditioning was obtained as a result of inactivating these cells by allostatin peptide treatment. This amnesia was reversed upon retesting the mice one day later without the allostatin peptides demonstrating the reversibility of the allostatin effects and the link between activity in the CREB cells and recall [22]. Despite the exclusive focus on CREB in the previous studies, the convergent findings using three different strategies strongly supports its important role in memory allocation in the amygdala.

Another influential factor that determines the allocation process appears to be neurogenesis in the dentate gyrus (DG). Using 5-bromo-2′-deoxyuridine (BrdU), a permanent stain that intercalates with dividing DNA allowing the tracing of newly born neurons, a recent study showed that 4- to 8-week-old DG neurons are preferentially recruited after spatial learning [28]. In contrast, 2-week-old neurons integrated with lower efficiency and 1-week-old neurons did not integrate at all [28]. In line with a recent study showing that 4-week-old (but not 1-week-old) neurons have the essential synaptic structure and physiology to support the appropriate connections with hippocampal circuits [29], this suggests that the timing of neuronal development relative to training is indeed vital in the memory allocation process. Nevertheless, the nature of memory allocation processes that take place in brain areas devoid of neurogenesis and outside the amygdala remains to be determined.

B. Memory Storage

After the initial allocation of a memory to a specific neural circuit begins the more prolonged process of systems consolidation that involves gradual reorganization of the brain regions that support memory formation and storage [13,14]. Classical studies characterizing memory loss in patients with lesions of the medial temporal lobe (MTL) [30,31] revealed that the hippocampus serves as a temporary store for new information, but that permanent information storage depends on a broadly distributed cortical network [14]. These human data are indeed consistent with observations that hippocampal lesions in the first week after training, but not

thereafter, disrupt contextual fear memories in rats, and thus, maintaining a proper hippocampal trace is crucial to establish remote memories in the cortex [32]. From more refined studies, several molecules have in the meantime been identified that maintain the hippocampal trace of a memory in the days following training for the existence of a remote memory [33,34] (for a more detailed overview of other molecules that are involved in memory storage, but not specifically assessed for remote memory storage, the reader is referred to [19]). For instance, when NMDA (N-methyl-D-aspartate) receptor (NMDAR) function was inducibly suppressed in the CA1 region in the week following the training of two hippocampal-dependent tasks (Morris Water Maze and contextual fear conditioning), remote memory formation for these tasks was blocked. However, when done at later time points, the suppression of the NMDAR function did not affect the remote memory formation [33]. Similar results were obtained when levels of α-calcium/calmodulin kinase II (α-CaMKII), a signaling enzyme mainly expressed in the excitatory neurons of the forebrain and essential for neuronal plasticity [35], were altered [34]: Overexpressing a dominant-negative form of α-CaMKII in the week after training, but not afterwards, blocked the formation of remote contextual fear memories [34]. Together, these results support the importance of the hippocampus (HPC), especially during the first week following encoding, for memory consolidation in cortical networks, and furthermore suggest that there is a crucial week-long window during which normal hippocampal activity is needed for the memory to be consolidated.

However, several studies found that cortical regions are also implicated in the initial phase of memory formation [36–39], thus challenging the idea that the HPC is solely involved in this process. In one of the recent studies in this regard [38], real-time optogenetic inhibition of excitatory medial prefrontal cortex (mPFC) neurons during contextual fear conditioning showed that such temporally precise inhibition impaired the formation of long-term associative memory, tested 30d after of acquisition [38]. In another recent study [39], using a doxycycline-inducible mouse line (TetTag) to tag the activated neurons [40], an optogenetic stimulation of the activated neural population during contextual fear memory training in the retrosplenial cortex (RSC), a cortical region implicated in episodic memories and emotional associations [41–44], was sufficient to produce fear memory retrieval even when tested 2d after acquisition [39]. These results are in line with previous studies [36,37] showing that the

prefrontal cortex (PFC) is critically involved in memory encoding, and that its inactivation by local infusion of NMDAR antagonist could block contextual memory acquisition in mice [36], and learning of new paired-associates in rats[37].

In another intriguing study, Lesburguères et al. used a social transmission of food preference (STFP) test, where an associative olfactory memory develops after a study animal (observer) learns about the safety of a certain food (novel odor for the observer) from an interaction session with another animal that has already tasted the food (demonstrator). Then the observer shows reduced fear towards this novel food upon the first encounter and significantly consumes it. The authors first showed that the acquisition of such food preference memory is dependent on the orbitofrontal cortex (OFC) only for 30d-old remote memory, but not for recent memory (24h after training), and that for the first period after training (7d) it is mainly HPC-dependent [45]. Nevertheless, the authors then went on to show that there is an intricate interplay between the HPC and the OFC for such memory to endure. Using the excitatory glutamate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) to block the activity of the OFC during the 2-week period following training, an unexpected memory loss to a novel odor test was observed 30d later. Likewise, inactivating the OFC immediately before training blocked the memory after 30d, and not after 7d, indicating that early cortical activity is required for subsequent stabilization of such memory [45].

Beyond memory formation, several studies investigated the role of extrahippocampal structures in remote memory storage, from which the anterior cingulate cortex (ACC) emerges to play a key role at least in remote contextual fear memory storage [46–49]. Thus, lidocaine-mediated pharmacological inactivation of the ACC disrupts the retrieval of remote contextual fear memory in mice 18d and 36d post training, while inactivating the prelimbic cortex (PL) – a region located near the ACC in the mPFC – at the same time points did not disrupt the very same memory [46]. Similarly, the lidocaine-mediated inactivation of the prefrontal cortex (PFC), and the ACC was shown to impair remote spatial memory retrieval when tested 30d after acquisition [47]. These results are in line with previously reported data from a study using non-invasive functional brain imaging to examine the metabolic activity of different brain regions underlying spatial discrimination memory storage in mice [48]. In this study, an increased metabolic activation in the frontal cortex, together with the recruitment

of the ACC and temporal cortices was observed 25d – but not 5d – post acquisition [48]. Together, these findings indicate a high level of involvement of cortical areas during the retrieval of remote memories, postulating these areas to be vital structures for remote memory storage.

Finally, from a reconsolidation point of view and how memory storage could affect such process, it has been previously demonstrated that infusing anisomycin (ANI), a protein synthesis inhibitor, to the dorsal HPC (dHPC) or the ACC after contextual fear memory recall (45d, or 30d post acquisition, respectively) disrupts the memory when tested 1d after anisomycin treatment [11,49]. Collectively, these results highlight an equal importance of hippocampal and cortical regions in remote memory reconsolidation, which suggest that probably memory formation and storage does not depend solely on a single brain area but is more distributed among different structures that share the upkeep of the trace.

II. Structural changes

Amongst many aspects that categorize a memory to be remote is persistence, yet it is still enigmatic how this property is achieved. The strength and number of synaptic connections that are formed after an experience offers one possible explanation as to how remote memories could endure and last throughout life [18] – since we know that such processes – such as increased dendritic spine density – are indeed implicated in 1d-old memories [15, 50,51]. In this section, we shed light on the structural changes that modify the connectivity of brain networks and that might underlie remote memory perseverance.

A few years ago, Ammassari-Teule and colleagues used contextual fear conditioning as a behavioral paradigm to show that recent and remote memory formation trigger region-specific and time-dependent morphological changes in hippocampal and cortical networks of mice [16]. Right after fear conditioning, there was a significant increase in spine density in the CA1 field of the hippocampus compared to the naïve or even pseudoconditioned groups. 36 days later, in contrast, this increase in spine density had developed sequentially when it reached the cortical regions, specifically the ACC. Thus, hippocampal plasticity per se is seemingly crucial in driving the

structural changes that were observed at a remote time point, yet its role was merely time limited, an observation that was recently confirmed using time-lapse two-photon microendoscopy [52]. To further prove this assumption, a hippocampal lesion was generated early at the day of conditioning, where it abolished the growth of significant spine density in the ACC (36d post-training) compared to the sham group [16]. In contrast, when this lesion was introduced at a later time point (24 days after conditioning), it did not prevent the spine density changes in the ACC neurons. The detected structural changes in either region were directly correlated to the strength of the conditioned memory: An absence of these structural in hippocampal or the cortical regions was accompanied by memory impairments for recent and remote memories, respectively. This is in line with a recent demonstration that such increase in synaptic density and plasticity occurs exclusively in engram cells, but not in non-engram cells, in the DG 24h after encoding [53].

Importantly, such structural remodeling in hippocampal and cortical regions is essential for memory stabilization and afterwards for remote memory expression. The spine growth at the hippocampal neurons is important at an early time point after conditioning, yet this importance starts to fade with time, when a more permanent trace is formed in the cortex [17], as illustrated by the following study. To inhibit the structural changes that occur in the cortex, a transcription factor that is known to negatively regulate spine growth, myocyte enhancer factor2 (MEF2) was overexpressed through a viral vector to increase the MEF2-dependent transcription in ACC neurons at 2 different time points, either 1 day or 42 days after conditioning. At the earlier time point, the stabilization of the conditioned memory and the associated increase in spine growth was blocked, whereas no effect was observed at the later time point [17]. This suggests that the increase in spine growth at the ACC following conditioning happens in a time-dependent manner and that it is central for the stabilization and persistence of such memory.

In contrast to the abovementioned studies, another study showed a rapid formation of new spines in the motor cortex of mice following a novel motor skill learning task [54]. Using in vivo superficial dendrites imaging, they demonstrated that there is an immediate formation of spines in the motor cortex following a novel motor learning task (within 1h after learning initiation), and that these spines are preferentially stabilized upon subsequent training and endure long after training stops (up to 120d)

[54]. This suggests that the early cortical structural changes during motor learning and the subsequent stabilization over months subserve as long-lasting structural basis for memory maintenance and persistence of a motor skill. Similarily, a more recent study reported that the encoding of a long-term episodic memory itself elicits early structural changes in neocortical regions. In this study, structural plasticity in the mPFC was significantly increased 1h following contextual fear conditioning [38]: Investigating the morphology of individual dendritic spines on mPFC pyramidal neurons revealed that the ratio between the thin spines to mushroom spines was significantly increased following conditioning. This suggests that dendritic spine plasticity in the mPFC circuit also contributes to memory encoding, which is surprising as the remodeling of the cortex was traditionally thought to be limited to the later stages of memory processing that promote remote memory storage [55]. Further investigations are now needed to have a better understanding of these structural changes and how they are employed to serve memory lasting, or extinction (Box 1).

III. Epigenetic regulation

Remote memories persist throughout the life of individuals, whereas the protein molecules that may subserve these memory traces are thought to turn over on the order of days [56]. To address such unanswered questions dealing with the molecular basis for a lifelong memory, it has been proposed by Francis Crick (1916-2004) in 1984, and later on by the molecular biologist Robin Holliday (1932-2014) in 1999, that epigenetic mechanisms – particularly DNA methylation – could partly explain the persistence of memories over a lifetime [57,58]. Epigenetics has long been heralded as a stable and self-perpetuating regulator of cellular identity through establishing persistent and heritable changes in gene expression across cell divisions[20]. Although the nervous system is essentially composed of non-dividing cells, the recent decade has shown that epigenetic mechanisms could nevertheless play a fundamental role in forming lasting memories.

Commonly, DNA is packaged into chromatin through its wrapping around octamers of histone proteins. Chromatin can exist either as heterochromatin, or as euchromatin:

Heterochromatin is characterized by condensed chromatin and subsequent transcriptional repression, whereas euchromatin is characterized by a relaxed chromatin state that allows the transcriptional machinery to access the DNA for gene expression [60]. Apart from short interfering RNA molecules that mediate posttranscriptional gene silencing [61] and induce epigenetic changes in gene expression via modifications of chromatin [62], the switch between both states of chromatin is governed by two major epigenetic modifications: DNA methylation, and post-translational modifications (PTMs) on histone tails. DNA methylation refers to the covalent addition of a methyl group to the cytosine base by DNA methyltransferases (DNMTs), while PTMs are the addition and removal of chemical moieties to histone tails, which are dynamically regulated by chromatin-modifying enzymes [22]. These modifications include – but are not limited to - histone acetylation, phosphorylation, and methylation [64] (see Tweedie-Cullen et al., for a complete overview of recently identified PTMs in the brain [65]). Both types of epigenetic modifications are associated with learning and memory, and many recent studies have shown that these epigenetic changes could support memory formation and maintenance through a cascade of specific changes to gene expression including enduring memories.

A. DNA Methylation

The first study to investigate the potential role of DNA methylation in regulating memory formation by Sweatt and colleagues showed that Dnmt gene expression is upregulated in the adult rat hippocampus following contextual fear conditioning, and that its inhibition blocks memory formation [66]. Accordingly, fear conditioning was associated with an upregulation of mRNA levels of the DNMT subtypes that are responsible for de novo methylation, DNMT3A and DNMT3B, in the CA1 region 30 min after training. Then, to show that the hippocampal DNMT activity is necessary for memory consolidation, DNMT inhibitors – 5-azadeoxycitidine (5-AZA) or zebularine (zeb) – were locally infused right after the training, where they abolished the freezing response of the injected group 24h after (test day 1). Interestingly, when retrained immediately after test day 1, and retested 24h later (test day 2), the DNMT inhibitor-treated group showed a significantly higher freezing than on test day 1, and

when retrained and retested 24 h later (test day 3), they showed an equivalent freezing to the vehicle-treated group. But when the 5-AZA was infused 6h after training, and animals were tested 18h later (24h after training), the inhibitor-injected group displayed normal fear memory indicating that the effect of DNMT inhibition is merely due to blocking consolidation and not due to any other effects on the retrieval or the performance of the animals [66]. These experiments suggest that the transient inhibition of DNMT in the hippocampus following training blocks memory consolidation in a resilient manner that could be reverted as soon as the inhibitor clears off and that the necessary DNA methylation states for consolidation could be re-established.

In a follow-up study, Miller et al. found a rapid increase in methylation of a memory suppressor gene in the hippocampal CA1 region 1h after contextual fear conditioning. Using quantitative real-time PCR, the methylation levels of protein phosphatase 1 (PP1), a memory suppressor gene that is suggested to promote memory decline [67], was dramatically higher in the fear-conditioned group compared to the control group. This increase in methylation was associated with lower levels of PP1 mRNA, yet the increase in methylation was attenuated and associated with a twofold increase in the mRNA levels when 5-AZA was infused locally 1h after training. Conversely, a demethylation of a memory-promoting gene was found in the CA1 region 1h after contextual fear conditioning. The demethylation of reelin, a gene that enhances long-term potentiation and the loss of function of which results in memory formation deficits [68,69], was pronounced in the trained group with its mRNA levels being significantly higher than the control group. DNMT inhibition using 5-AZA led to further demethylation of reelin and even higher levels of its mRNA. These data suggest that the DNA methylation is dynamically regulated and that it is a crucial step in memory formation.

Importantly, cortical DNA methylation also seems to support remote forms of memories [70]. The cortical DNA methylation of the memory suppressor calcineurin (CaN, also known as Ppp3ca), a gene that downregulates pathways supporting synaptic plasticity and memory storage was investigated using methylated DNA immunoprecipitation (MeDIP) in rats. CaN's cortical DNA methylation persisted for at least 30d after contextual fear conditioning, and its mRNA levels were significantly reduced in the trained group 2h after retrieval 30d post-training. Importantly, when

the NMDA receptor antagonist (AP5) was infused into the dorsal hippocampus (CA1) just before training, CaN methylation in the dorsal medial prefrontal cortex (dmPFC) 7d after training was blocked, indicating that a single hippocampus-dependent learning experience is sufficient to drive lasting, gene-specific methylation changes in the cortex. Moreover, intra-ACC infusions of DNMT inhibitors (5-AZA, or zeb, or RG108) 30d after training disrupted fear memory and was associated by a significant reduction in the CaN methylation levels. However, the infusion of these inhibitor 1d after training had no effect on fear memory 30d later [70]. These results indicate that cortical DNA methylation is indeed triggered by a learning experience, and most importantly, its perpetuation supports long-lasting, persistent memories. More detailed studies including investigating DNA methylation changes on a genome-wide scale or within engram-bearing cells are clearly warranted to deepen our knowledge of the implication of these changes in remote memory storage.

B. Histone PTMs

Newly formed hippocampus-dependent memories need to be stabilized into a long-lasting ACC-dependent memory trace [46,71,72]. Several studies demonstrated changes in gene expression in both brain regions accompany such stabilization [46,47]. This differential gene expression has recently been associated with epigenetic modifications in terms of histone PTMs [73]. Using a novel object recognition task on mice, serine (S) 10 phosphorylation on histone (H) 3, lysine (K) 14 acetylation on H3 as well as H4K5 acetylation, and H3K36 trimethylation in the PFC associated with remote (7d post training) memory consolidation. Importantly, the doxycycline-inducible selective inhibition of the memory suppressor gene PP1 in a transgenic mouse line showed improved remote memory performance accompanied by increased histone PTMs. In contrast, blocking the occurrence of these PTMs using a cocktail of inhibitors targeting the epigenetic enzymes responsible thereof impaired remote object memory, suggesting that these histone PTMs are essential for memory consolidation and retention. Finally, these histone PTMs were increased in the promoter region of Zif268 – an immediate early gene important for memory formation and storage [74] – and that its expression levels shifts from the hippocampus to the PFC as the memory matures [73]. This study shed light on the

spatiotemporal dynamics of these histone PTMs in the hippocampus and cortex and demonstrated that they could act as molecular marks subserving memory consolidation – at least up to 7d post-training.

Similar results were obtained for memory consolidation of social transmission of food preferences [45]. There, associative olfactory memory was linked to a marked increase in H3 acetylation in the OFC 1h after training, but such increase disappeared upon inactivating the OFC using tetrodotoxin or CNQX. Additionally, increasing the OFC histone acetylation by infusing HDAC inhibitors (sodium butyrate or Trichostatin A) was associated by an increase in memory robustness at the remote timepoint (30d) [45]. Together, these results stipulate that this cortical epigenetic mark observed very early during training might be essential for tagging these neurons to allocating them to the long-term olfactory memory and that thereafter, these neurons will participate in the system consolidation process driven by the HPC-OFC circuitry in order to help this memory to endure. It would be highly interesting to repeat this study with CREB-transfected OFC neurons in order to test this hypothesis.

In addition to histone PTMs, a recent study by Zovkic et al. has shown that a variant of histone H2A (H2A.Z) is actively exchanged in the hippocampus and cortex in response to fear conditioning in mice [75]. H2A.Z is known to be associated with nucleosomes adjacent to the transcription start site (TSS) of a gene, and its presence has been strongly linked to dynamic changes in gene expression [76]. To investigate its effect on transcriptional changes associated with learning, chromatin immunoprecipitation (ChIP) was used. Binding of H2A.Z was reduced at the +1 nucleosome (first nucleosome downstream of the TSS) of memory-promoting genes (Npas4, Arc, Egr1, Egr2, and Fos), and there was an increase in the expression of those genes 30 min after the contextual fear training. In contrast, H2A.Z binding was increased for the memory-suppressor gene CaN and associated with reduced expression of this gene. This suggests that H2A.Z at the +1 nucleosome restricts memory-related gene transcription [75]. Furthermore, the methylation of the promoter region of the gene encoding H2A.Z (H2afz) was shown by MeDIP to be increased 30 min after contextual fear conditioning, when it was accompanied by reduced H2A.Z protein expression throughout the hippocampus, whereas the expression levels of H2A.Z returned to baseline after 2h [75].

To assess a causal involvement of H2A.Z in memory consolidation, an adeno-associated virus (AAV) depleting H2A.Z in the dorsal CA1 region of the hippocampus was used. This approach improved fear memory 24h and 30d after training compared to a scramble-injected control group. In contrast, when H2A.Z was depleted from the mPFC, there was no effect on fear memory at the hippocampus-dependent 24h time point, yet the freezing was significantly higher at remote time points 7 and 30 days post-training [75]. Moreover, a genome-wide transcriptional analysis was carried out to evaluate the impact of H2A.Z depletion on training-induced gene expression in CA1 and mPFC 30 min after training. The analysis showed a differential expression – between the trained and untrained groups – in many genes including a number of the early learning-related genes; Arc, Fos, Egr1, and Egr2 [75]. Although the study did not ascertain the specific target genes through which H2A.Z regulates memory, it clearly demonstrated that H2A.Z is dynamically regulated during learning and memory and that it could be an important epigenetic contributor to the complex coordination of gene expression in memory. Future, more refined studies will certainly help to elucidate the role of histone exchange and histone PTM processes associated with remote memory storage, or extinction (Box 2).

SUMMARY

The allocation of a memory to a particular neural circuit is a critical step in memory formation. We reviewed how CREB is involved in such process highlighting its important role. Additionally, electrophysiological studies showed that cells transfected with CREB viral vectors are more excitable compared to the neighboring cells or even those transfected with the control vector [22]. This could partially address the preference of allocating the memory to CREB cells since their increased excitability might render them more responsive to sensory inputs and therefore more likely to get activated during conditioning training. However, it could still be possible that there are other molecular determinants and processes that are important for memory allocation and, although ubiquitously expressed, it seems unlikely that memory allocation depends solely on CREB. Likewise, adult neurogenesis is restricted to only certain brain regions, and the data showing that new granule cells when mature are increasingly likely to be incorporated into circuits supporting spatial

memory [28,29] is not necessarily the sole determinant of allocating a memory to a specific neural population.

Another important aspect of memory persistence is which brain regions maintain its storage and what supports such perseverance. We highlighted the importance of the ACC in the upkeeping of remote memories since its inactivation prevents the recall of remote contextual fear memory as well as the reconsolidation of such remote memory 24h after its retrieval [46,49]. Intriguingly, a recent study identified for the first time monosynaptic projections from the ACC to the hippocampal CA fields that controls memory retrieval in mice [77]. Using retrograde tracers, this study characterized novel connections between ACC and CA fields (AC-CA) that subserve a potential bidirectional communication between the ACC and the hippocampus. Manipulating these projections optogenetically demonstrated a causal top-down control on memory retrieval, where the cells contributing to the AC-CA projection can activate contextually conditioned fear behavior (3d old memory), whereas their inhibition impaired the retrieval of such memory [77]. Nevertheless, further investigations are still needed to elucidate the role of these projections on the regulation of different memory processes.

Indeed, the cellular reconsolidation of a remote memory might not solely depend on the ACC since it has been shown previously that infusing anisomycin in the dHPC blocks the reconsolidation of remote contextual fear memory and that optogenetically inactivating the CA1 region would even impair recalling it [12]. Contradictorily, another study did not find any evidence that neither of the ACC nor the dHPC are involved in the cellular reconsolidation of remote contextual fear memory following retrieval [78]. More studies are highly anticipated to resolve these divergent findings, although such discrepancy could be partly attributed to the difference in the strength and length of the training and retrieval sessions used or in the inactivation method and its efficiency, since it has been demonstrated that these experimental conditions significantly affect the behavioral outcome [10,79].

Structural plasticity is another key point towards understanding the endurance of some memories. It provides a physical substrate for the storage of memories. We highlighted the synaptic plasticity that follows memory formation at hippocampal dendrites, and that such plasticity reaches cortical areas in a time-dependent manner

[16,17]. Nonetheless, we also shed light on two interesting studies supporting the view of an early cortical reorganization during motor skill learning [54] as well as episodic memory acquisition [38], which demonstrated the importance of such structural changes for lasting memories. The reduced density of spines in cortical areas upon remote fear extinction is in line with these findings and suggests a remodeling in the cortical circuit of the original memory [80]. However, a contradicting study showed that it is rather fear memory formation that is accompanied by spine elimination and that extinction involves spine formation [81]. These results are quite confusing, and although they could also be reflecting that opposite processes are at play in different cortical areas, they need to be addressed properly soon.

The epigenetic regulation was the final point we highlighted in this review, and the data we reviewed – collectively – support a dynamic pattern of epigenetic modifications including both DNA methylation [70], and histone PTMs [73] that subserve a spatiotemporal shift of the memory trace from the HPC to higher cortical regions during the process of memory consolidation. Also, the early tagging of certain neurons with epigenetic marks during encoding is central for the memory to be allocated to the tagged neurons and for the subsequent participation of these neurons in the circuit supporting such memory [45]. Furthermore, the extinction of remote fear memories with an HDAC2i increased histone acetylation-mediated neuroplasticity [82], and the lack of such plasticity from the hippocampus upon remote memory recall supports the idea of hippocampal disengagement for remote memories [46,48,55]. Nevertheless, it remains unclear whether memories might indeed be "coded in particular stretches of chromosomal DNA" as originally proposed by Crick [57], and if so, what the enzymatic machinery behind such changes might be. In this regard, cell population-specific studies are highly warranted.

Taken together, we find ourselves in an exciting period witnessing an increasing number of studies, which dare to investigate remote memory formation, storage and persistence. Yet it is clear that we are still in need of further investigations to unveil the dynamics of neuronal circuits and molecular mechanisms mediating such persistence. Ultimately, deciphering these processes would definitely contribute to the understanding, and possibly dulling, of abnormally long-lasting fear memories like those underlying anxiety disorders or post-traumatic stress disorder..

Box 1: Recent insights into structural plasticity and remote fear memory extinction

In addition to remote memory storage, memory extinction – in the case of remote fearful memories – also alters structural spine plasticity. For instance, remote memory extinction was found to diversely alter the spine density and spine size in the ACC and infralimbic cortex (ILC) in mice [80]: Extinction of a 31d-old contextual fear memory decreased the density of dendritic spines in the ACC significantly, but not the size. In contrast, the spine density remained elevated in the ILC but the size of spines decreased dramatically. The persistence of spine enlargement in the ACC upon extinction could be essential to warrant that the consolidated fear, as well as the extinction memory traces are kept in a dormant state to allow their reactivation long after training. This may indicate that the extinction per se partially remodels the neuronal network supporting the original memory representation.

Lately, a study described opposing effects of fear conditioning and extinction on dendritic spine remodeling in the frontal association cortex (FrA) of rats [81]. Using two-photon microscopy to examine the formation and elimination of postsynaptic dendritic spines of the FrA, the cued fear conditioning caused rapid and long-lasting spine elimination that was significant over 2 and 9 days. After 2 days of extinction training, the spine formation was significantly increased and its degree predicted the effectiveness of the extinction to reduce the conditioned freezing response. These results paradoxically conclude that fear conditioning mainly promotes spine elimination, whereas extinction essentially induces spine formation. More studies in different brain areas will be of high interest to corroborate these findings.

Box 2: Recent insights into epigenetic dynamics of remote memory attenuation

In addition to memory formation and storage, a recent study also showed an epigenetic involvement into remote fear memory attenuation [82]. In this study, permanent attenuation of remote fear memories was achieved by using a histone deacetylase-2 inhibitor (HDAC-2i) in combination with reconsolidation-updating paradigms, which increased the acetylation levels of histone H3K9/14 (AcH3). In contrast to a vehicle-treated control group that was resistant to remote memory attenuation, a significant increase in AcH3 was noticed 1h post-recall in the ACC, which stayed elevated even after the extinction training. In the HPC, no change was observed in the acetylation levels of AcH3 1h post-recall, yet a significant increase was seen in the HDAC-2i-treated group after extinction training. More specifically, this observed increase in acetylation in the HDAC2i-treated group was detected in the promoter region of neuroplasticity-related genes such as cFos, Arc, and Igf2, which showed a concomitant increase in expression [83]. This clearly displays that extinguishing remote fear memories using an HDAC2i promotes increased histone acetylation-mediated neuroplasticity, and in turn demonstrates an epigenetic contribution to remote fear memory attenuation.

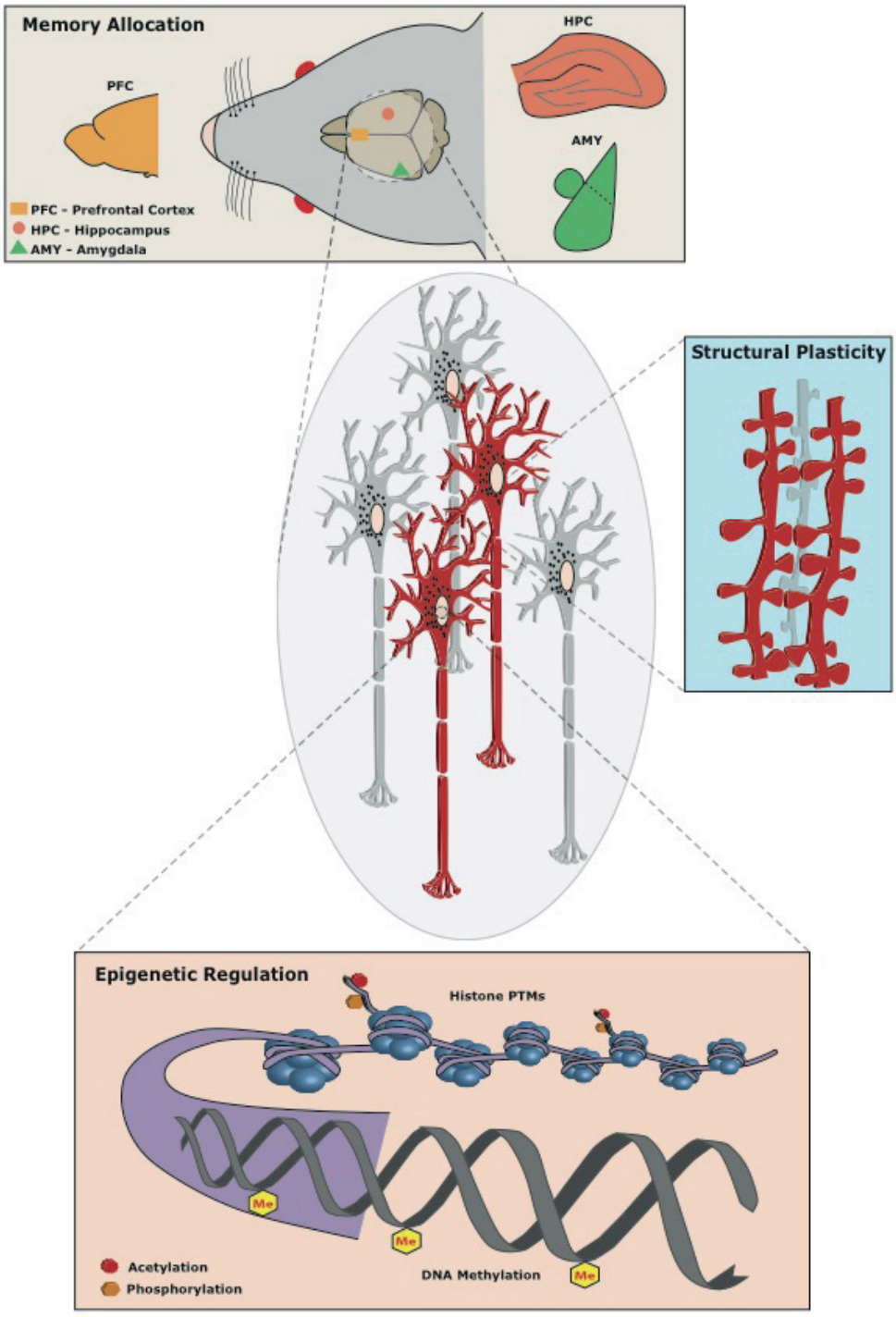

Fig.1 : Schematic illustrating three essential mechanisms that might contribute to remote memory storage and thus, memory endurance in the (rodent) brain, which are discussed in this review. First, during memory allocation, learning induces the activity of a specific subpopulation of cells – likely spread across different brain areas – which will become recruited into the memory trace. The amygdala (AMY), the hippocampus (HPC) and the prefrontal cortex (PFC) are known to be activated during memory allocation (for details see text). Second, in cells allocated to a specific memory – also known as the memory engram [1-3] – structural changes at the level of dendritic spines have been demonstrated by several studies. These changes are exclusive to the cells of the memory trace or engram (red), but not observed in other cells (grey). Third, memory engram cells are also likely to be

characterized by epigenetic changes, such as posttranslational modifications (PTMs) on histone proteins, and methylation of the DNA, the core chromatin constituents. Note, however, that such engram-specific engagement of epigenetic mechanisms remains to be experimentally demonstrated.

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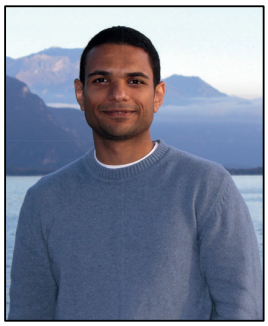

SUMMARY

A citizen of Earth who has been procreated in Cairo, Egypt. Grew up in the middle of an imposing civilization to become an apothecary, a neuroscientist, and a pilgrim of science.

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EDUCATION

Swiss Federal Institute of Technology (EPFL)
Doctorate in Neuroscience

Thesis project on remote traumatic memories
Lausanne / 2012 – till date

American University in Cairo (AUC)
Master in Biotechnology

Thesis project on Neurogenetics at UT Southwestern Medical center
Cairo & Dallas / 2008 – 2012

Helwan University
Bachelor of pharmacy
Magna cum laude
Cairo / 2000 – 2005

EXPERIENCE

Swiss Federal Institute of Technology (EPFL)
Doctoral Assistant – Brain Mind Institute
Lausanne / October 2012 – till date

UT Southwestern Medical Center
Junior Research Fellow – Hiesinger's Fly Lab
Dallas / July 2011 – October 2012

American University in Cairo (AUC)
Teaching Assistant – School of Sciences and Engineering
Cairo / September 2008 – June 2011

National Research Center (NRC)
Research Assistant – Industrial pharmacy Lab
Cairo / April 2006 – till date (on sabbatical)

Seif Pharmacies
Pharmacy Manager
Cairo / October 2005 – February 2007

AWARDS

Science Slams
1st prize
Helsinki / November 2016
Berlin / March 2017

Swiss FameLab
1st prize - Audience & Jury Awards
Zurich / May 2016

Hall of FameLab
1st prize – Audience & Jury Awards
Natural History Museum, London / October 2016

ENCODS Neuroscience Conference
Best Talk Award
Sesimbra, Portugal / April2015

PUBLICATIONS

O. Khalaf and J. Gräff - *Structural, Synaptic and Epigenetic Dynamics of Enduring Memories* Neural Plasticity, volume 2016 - p. 3425908, 2016

O. Khalaf, B. Fauvet, A. Oueslati, I. Dikiy, AL. Mahul-Mellier, F.S. Ruggeri, M.K. Mbefo, F. Vercruysse, G. Dietler, S.J. Lee, D. Eliezer, H.A. Lashuel - *The H50Q mutation enhances alpha-synuclein aggregartion, secretion, and toxicity,* Journal of Biological Chemistry, 2014; 289 (32): 21856-76

D. Wang, D. Epstein, **O. Khalaf**, S. Srinivasan, W.R. Williamson, A. Fayyazuddin, F.A. Quiocho, P. R. Hiesinger - *Ca^{2+}-Calmodulin regulates SNARE assembly and spontaneous neurotransmitter release via v-ATPase subunit V0a1,* Journal of Cell Biology, 2014; 205 (1): 21-31

SUPERVISED PROJECTS

Characterization of neuronal population of successful attenuation of remote fear memory
Siegfried Resch - Masters thesis, 2015
University of Applied Sciences – Austria

Identification of neuronal population of remote fear extinction using catFISH
Victoire Gorden- Masters thesis, 2017
EPFL – Lausanne

EXTRACURRICULAR ACTIVITIES

Toastmasters Club President
EPFL-UNIL club
July 2016 – till date

Science Communication Mentor
The British council SchoolLab competition
Boulogne-Sur-Mer/ March 2017

The "Biotech Wave" Newsletter
Founder and Editor
AUC - Cairo / 2008 - 2011

Parachuting Instructor
Static-line parachuting
Cairo/ 2002-2003

LANGUAGE SKILLS

Arabic: Mother tongue
English: Fluent
French: Proficient

PERSONAL DATA

Nationality: Egyptian
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Status: Single

Last updated March 2017