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Neural stem cell expansion rejuvenates learning strategies and memory throughout life

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SUMMARY

The hippocampus is a brain area fundamental for the generation of conscious memories and the establishing of flexible relationships between contextual representations. During aging, hippocampal malfunction has been proposed to be a key factor leading to cognitive decline, and understanding the potential mechanisms that could prevent or reverse these impairments has become imperative in the context of a rapidly aging population.

The hippocampus is one of the only two areas in the brain where new neurons are constantly generated during adulthood. Yet, their role in learning and memory functions is not well understood. Interestingly, adult hippocampal neurogenesis has been proposed as a cellular component of a brain reserve mechanism with the potential to preserve cognitive abilities throughout life, as well as a putative target for therapeutic approaches.

In this work, I used a specific, genetically-driven expansion of hippocampal neural stem cells to exploit their intrinsic potential to generate newborn neurons, leading to increased neurogenesis throughout life. This promoted hippocampal function at several levels, improving flexible learning in navigational tasks during youth, compensating age-related cognitive decline and rejuvenating contextual memory during aging.

Altogether, my work provides a better understanding of the functional contribution of neurogenesis to learning and memory and demonstrates that critical aspects of hippocampal cognitive impairment can be reversed in old age or compensated throughout life by extrinsically exploiting endogenous brain reserves.

ZUSAMMENFASSUNG

Der Hippocampus ist ein Hirnareal welches fundamental für die Generierung von bewussten Erinnerungen und für die Etablierung von flexiblen Beziehungen zwischen kontextuellen Repräsentationen ist. Hippocampale Fehlfunktion während des Alterns wurde als ein Schlüsselfaktor für den kognitiven Abbau vorgeschlagen und im Kontext einer schnell alternden Bevölkerung wird es zwingend notwenig, mögliche Mechanismen zu verstehen, die diese Einschränkungen verhindern oder rückgängig machen können.

Der Hippocampus ist ein von nur zwei Arealen im Gehirn, in dem neue Neuronen ständig im Erwachsenenleben erzeugt werden. Deren Rolle beim Lernen und bei Gedächtnisfunktionen ist jedoch nicht gut verstanden. Interessanterweise wurde adulte hippocampale Neurogenese als eine zelluläre Komponente eines Gehirnreservenmechanismus vorgeschlagen, mit dem Potenzial kognitive Fähigkeiten ein Leben lang zu erhalten sowie ein mögliches Ziel für therapeutische Ansätze darzustellen.

In dieser Arbeit habe ich eine spezifische, genetisch-bedingte Expandierung von hippocampalen Nervenstammzellen genutzt, um deren intrinsisches Potenzial, neugeborene Neuronen zu erzeugen, auszuschöpfen, was zu einer lebenslangen erhöhten Neurogenese geführt hat. Dies hat die hippocampale Funktion auf mehreren Ebenen gefördert, vom verbesserten flexiblen Lernen in Navigationsaufgaben in der Jugend, über Kompensation des altersbedingten kognitiven Abbaus bis hin zur Verjüngung von kontextuellem Gedächtnis beim Altern.

Zusammengefasst stellt meine Arbeit ein besseres Verständnis des funktionellen Beitrags der Neurogenese zu Lernen und Gedächtnis zur Verfügung and zeigt, dass kritische Aspekte hippocampaler kognitiver Beeinträchtigung im Alter rückgängig gemacht oder ein Leben lang durch extrinsische Ausnutzung der endogenen Hirnreserven kompensiert werden können.

Übersetzt von Simon Hertlein

Precious Memories

As I travel down life's pathway,
know not what the years may hold.
As I ponder, hopes grow fonder,
precious memories flood my soul
Precious father, loving mother,
glide across the lonely years.
And old home's scenes of my childhood
in fond memory appears.
Precious memories, how they linger
how they ever flood my soul.
In the stillness of the midnight,
precious sacred scenes unfold.

Bob Dylan
(adapted from J.B.F. Wright)

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ABBREVIATIONS

4D	cdk4/cyclinD1
BLA	basolateral amygdaloid nucleus
BrdU	5-bromo-2'-deoxyuridine
CA	*cornu ammonis*
CeA	central amygdaloid nucleus
DAPI	4'-6-diamidino-2-phenylindole
DCX	doublecortin
DG	dentate gyrus
EdU	5-ethynyl-2-'deoxyuridine
EC	entorhinal cortex
GCL	granular cell layer
GFP	green fluorescent protein
HIV-1	human immunodeficiency virus type-1
IEG	immediate early gene
IPs	intermediate progenitors
LTM	long-term memory
LTP	long-term potentiation
ML	molecular layer
NLS	nuclear localization signal
NSC	neural stem cell
OR	odds ratio
SGZ	subgranular zone
STM	short-term memory
SVZ	subventricular zone
TAM	tamoxifen
VSV-G	vesicular stomatitis virus type-G

CHAPTER 1

INTRODUCTION

1.1 MEMORY SYSTEMS IN THE MAMMALIAN BRAIN

A brief historical perspective

Learning and memory are the basic abilities of the nervous system to acquire, store and retrieve information. Creating memories is the most fundamental cognitive function of the brain. It allows us to stretch the instant in time where we live by remembering the past, as well as imagining the future. All life is memory, except for the one present moment, and the dream of the future yet to be lived is based on our previous learnt experiences. Memory is essential for survival, and all animals learn and create memories in one way or another. In its simplest form, a learnt reflex can help invertebrates to avoid a negative stimulus in the future. On a greater level of complexity, memories of past experiences can modify sophisticated behaviours like hunting, finding shelter or communicating. In humans, our memories define ourselves, and losing them leads to oblivion of who we are.

The nature of memory is a recurrent topic in philosophy and early psychology (Radvansky, 2017). Unlike Ancient Egyptians who associated the mind to the heart, Plato situated the mind in the brain. In the rationalist Platonic-thinking, memory served as the bridge between the perceptual and the idealized world, viewing the mind as an inner artist who paints our unreliable external perceptions on the soul. Plato's metaphor of memory as a wax tablet where images of the natural world are imprinted remains familiar to our daily experience of memory, and introduced elements like encoding efficiency (the pattern of the impression), retrieval and forgetting (the ability to read those impressions). At odds with his teacher, Aristotle defined memories as passive associations between stimuli or experiences, beginning a long tradition of empiricism whose most famous example is the study of classical (or Pavlovian) conditioning in the late 19th century. Curiously, these different views about the active or passive role of the brain in making memories and, ultimately, interpreting the world, lasts until our days (Buzsáki and Llinás, 2017; Llinás and Paré, 1991).

Experimental psychologists played a prominent role in the development of memory research for the last 150 years. Karl Lashley, for instance, is well known for his lesion studies in the search of the physical trace of the memory (i.e. engram, a term coined by Richard Semon in 1921; Josselyn, 2010). After years of infructuous experiments removing big cortical areas from rodents before and after learning, he said: "*I sometimes feel, in reviewing the evidence on the localization of the memory trace, that the necessary conclusion is that learning is just not possible*". Nowadays, we know that memory is not stored in a single area of the brain, but rather it is a highly distributed process. Other early memory researchers like Ebbinghaus and Bartlett were important in defining terms like learning and forgetting curve, and in pointing out the reconstructive nature of memory based on observational studies across the lifespan (Radvansky, 2017). In 1900, Müller and Pilzecker introduced the term *consolidation* for the

strengthening of learnt associations, and proposed retroactive interference between recent memories as a mechanism for wrong memory recall (Müller and Pilzecker, 1900), providing important insights that have guided memory research for over 100 years (McGaugh, 2000). In parallel, eminent neuroanatomist Ramon y Cajal proposed the synapse as the functional unit of the nervous system, and changes in the strength of these contacts as the cellular substrate of learning (Ramón y Cajal, 1894). This idea was refined by Hebb (1949), the father of computational neuroscience, and it is summarized in the sentence "*neurons wire together if they fire together*" (Lowel and Singer, 1992, page 211). Hebb postulated that the encoding of new memories occurs in a two-stage process, from the activation of neural assemblies to the stabilization of their interconnections. This reinforced the old notion that memory exists in different forms, defined as immediate or short-term memory (STM) and lasting or long-term memory (LTM; Atkinson and Shiffrin, 1968; James, 1890; McGaugh, 1966). Soon after, the discovery of long-term potentiation (LTP; Bliss and Lømo, 1973) in hippocampal slices provided a solid biological mechanism for Hebb's postulate, and is widely considered to constitute a basis for sustained synapse modifications and memory storage (Nicoll, 2017; **Fig. 1.1**).

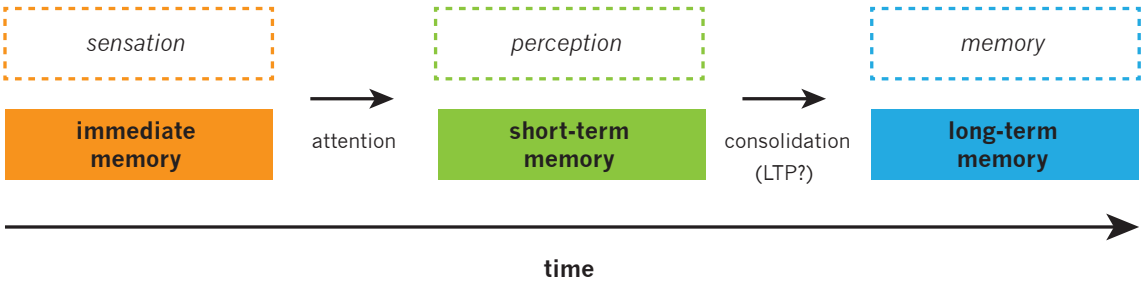

Fig. 1.1: Modal model of memory (modified from Atkinson and Shiffrin, 1968).
Scheme integrating historical terms used to describe mnemonic function (dashed boxes) with the current model to explain memory storage in the brain in sequential compartments. Different processes link these compartments, like attention and consolidation, for which LTP has been proposed to provide a plausible biological mechanism.

Memory systems: discovery and classification

In 1953, Henry Molaison (often referred to in the literature as H.M.) became one of the most important patients in cognitive neuroscience after the resection of his medial-temporal lobes that took away his ability to create new memories, together with his epileptic seizures. Posterior evaluation of this and other cases demonstrated the important role of the hippocampal region in normal memory function (Scoville and Milner, 1957). H.M. was studied for several years by many doctors who determined that his amnesia was mainly restricted to new autobiographical episodes, while his ability to acquire motor skills remained unaffected (Corkin, 1968; Dossani et al., 2015). This confirmed the existence of memories of different nature (a concept that traces back to the time of Saint Augustine) and the anatomical distribution of those memories in the brain.

Memory has been traditionally divided in two major categories: declarative (i.e., explicit or consciously recollected) and non-declarative (i.e., implicit or unconscious; reviewed in Cohen and Squire, 1980; Eichenbaum and Cohen, 2001; Mayford et al., 2012; Milner et al., 1998; Squire, 1986, 2004; Squire and Dede, 2015), from which three main memory systems can be defined (McDonald and White, 1993; Squire et al., 2012). The **declarative memory system** refers to what we normally think about when we talk about "memory". It is phylogenetically recent and encodes information about general facts (semantic memory) and autobiographical episodes (episodic; see Tulving, 1972 for a detailed definition). In a broader sense, it stores the relationship among items and events by making internal representations that model the external world. This is the memory system impaired in amnesic patients like H.M., and is highly dependent on the medial-temporal brain lobes where the hippocampus is located. On the other hand, non-declarative, unconscious forms of memory are unaffected in amnesic patients and cannot be expressed with words. This kind of memory is phylogenetically older

and can be divided in **procedural** and **emotional memory systems.** The former mainly refers to habits and motor skills, depends on striatal function and improves behavioural performance upon repetition, like for instance learning how to ride a bike. The latter mediates preference and aversion, and the physiological responses to them, involving the amygdala. A schematic representation of these main memory systems, examples and the relations among them can be seen in **Fig. 1.2**.

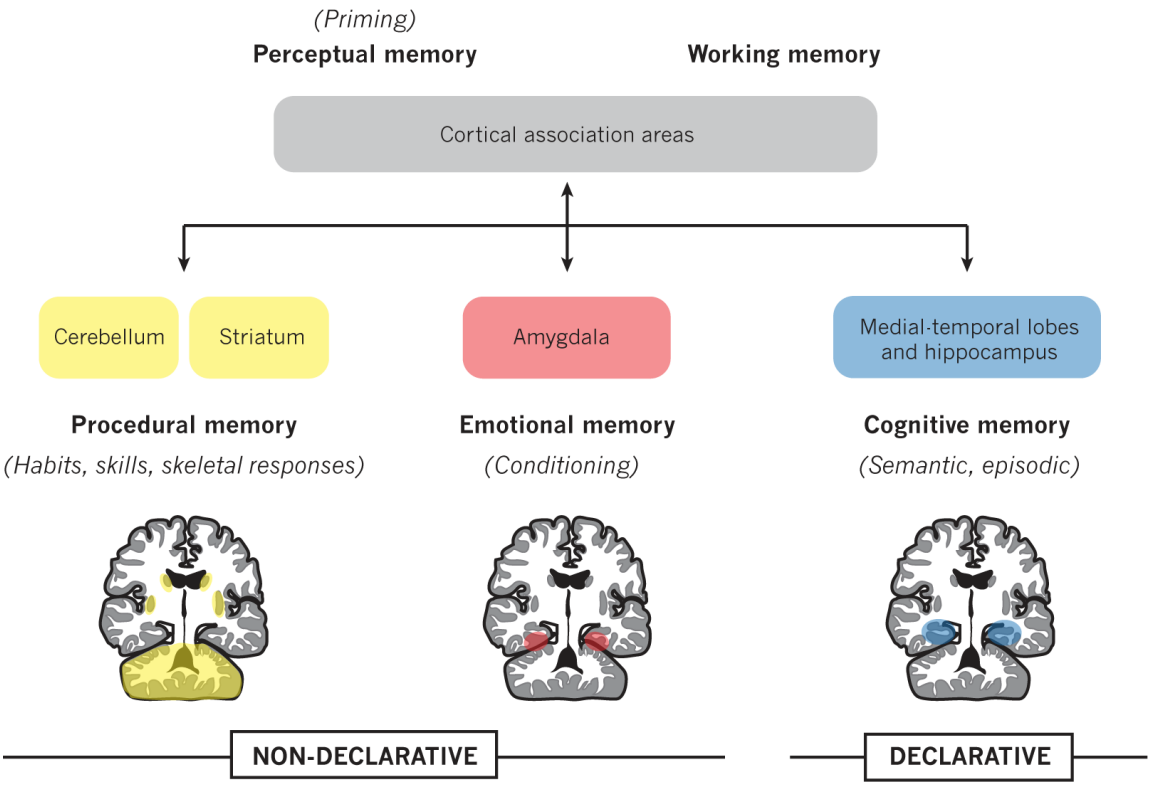

Fig. 1.2: Memory systems in the brain (adapted from Squire et al., 2012).
Organization of long-term memory systems in the mammalian brain with the structures thought to be important for their function. Declarative forms of memories are episodic (remembering having read a book last month) and semantic (knowing that the Earth is the third planet of the Solar System). Examples of non-declarative forms of memory are improvements in visual recognition of items upon repetition (priming, cortex-dependent), learning how to play piano (skill), sensory-motor adaptations of skeletal muscles (involving the cerebellum) and the fear associated to a bad experience (emotional memory). In many cases, the cortex is the final destination of long-term memories (Frankland and Bontempi, 2005; Squire et al., 2012).

This way, memory systems are different in the nature of the memory representation, its organization and the flexibility of its expression (Squire et al., 2012). Thus, memories could be defined as the changes, through the process of learning, of the neural networks integrating these systems.

Interactions between memory systems

While sensory pathways like vision, audition, etc., are vastly segregated in the brain, memory systems are blind to these distinctions having all of them access to all modalities of information through connections to highly associative cortical areas (Squire et al., 2012). Although evolution might have favoured the specialization of memory systems for independent processing (Sherry and Schacter, 1987), they are highly interactive and interconnected. As a consequence, information arising from the same episode can be processed in parallel by more than one system and reinforce each other. Strong emotional events, for example an unpleasant encounter with a snake, are more vivid, longer remembered and easier to recall (Buchanan, 2007; Holland and Kensinger, 2010), which shows cooperation between memory systems. Alternatively, the episodic component (the *what*, *where* and *when*) can be extracted and recalled independently of the fear associated to it. In a similar way, the encounter can cause a traumatic fear to snakes even if the event itself has been forgotten.

Examples of interactions between memory systems: fear conditioning

Experimental behavioural tasks aim to model these real-life situations, allowing the study of interactions between memory systems. Further elaborating on the previous example, **fear conditioning** tests are broadly used paradigms in which animals learn to associate one or more stimuli to fearful responses (**Fig. 1.3**). The

amygdala, a sub-cortical component of the limbic system that is key for emotional processing, integrates a variety of inputs from different cortical areas and sends projections to response centres that mediate the expression of the emotion (in this case, fear) through interactions with the autonomous nervous system that lead to sweating, changes in heart rate or motor, freezing responses (Davis, 1992; Kim and Jung, 2006; LeDoux, 2000). Different lesion and stimulation experiments have revealed the locus of the fear memory, the area where the emotional association through synaptic strengthening is made, as the amygdala (reviewed in Kim and Jung, 2006; Maren and Quirk, 2004). Depending on the nature of the conditioned stimulus the involvement of different structures on the emotional response can be studied. In unimodal associations like auditory-cue conditioning the emotional memory system operates alone (classical Pavlovian conditioning); if the stimulus associated to the fear response is multimodal and episodic, the emotional and declarative memory systems will be interacting (**Fig. 1.3**; Squire et al., 2012). In contextual conditioning paradigms fear is associated to the presentation of contexts (defined as the spatial circumstances around an event, encoded by the hippocampus; Fanselow, 2000; Maren et al., 2013) and an efficient interaction between both the declarative and emotional memory systems is necessary for memory retrieval in rodents and humans (Bechara et al., 1995; Phillips and LeDoux, 1992; Selden et al., 1991).

Recently, experiments have shown that artificial manipulation of engrams associated to different contexts is sufficient for emotional memory expression and the inception of false memories in the mouse brain (Ramirez et al., 2013), underscoring the strong influence of the hippocampal, contextual input on the creation of emotional memories. On the other hand, the amygdala can also help to consolidate declarative memories with strong emotional valence (a typical example is remembering vividly how you first learnt about the terrorist attacks of September 11th in New York in 2001, but not what you had for lunch the same

day; Buchanan, 2007; Holland and Kensinger, 2010; McGaugh, 2000; McGaugh and Roozendaal, 2009), as an adaptive mechanism of the brain to store information that is worth remembering (Squire et al., 2012).

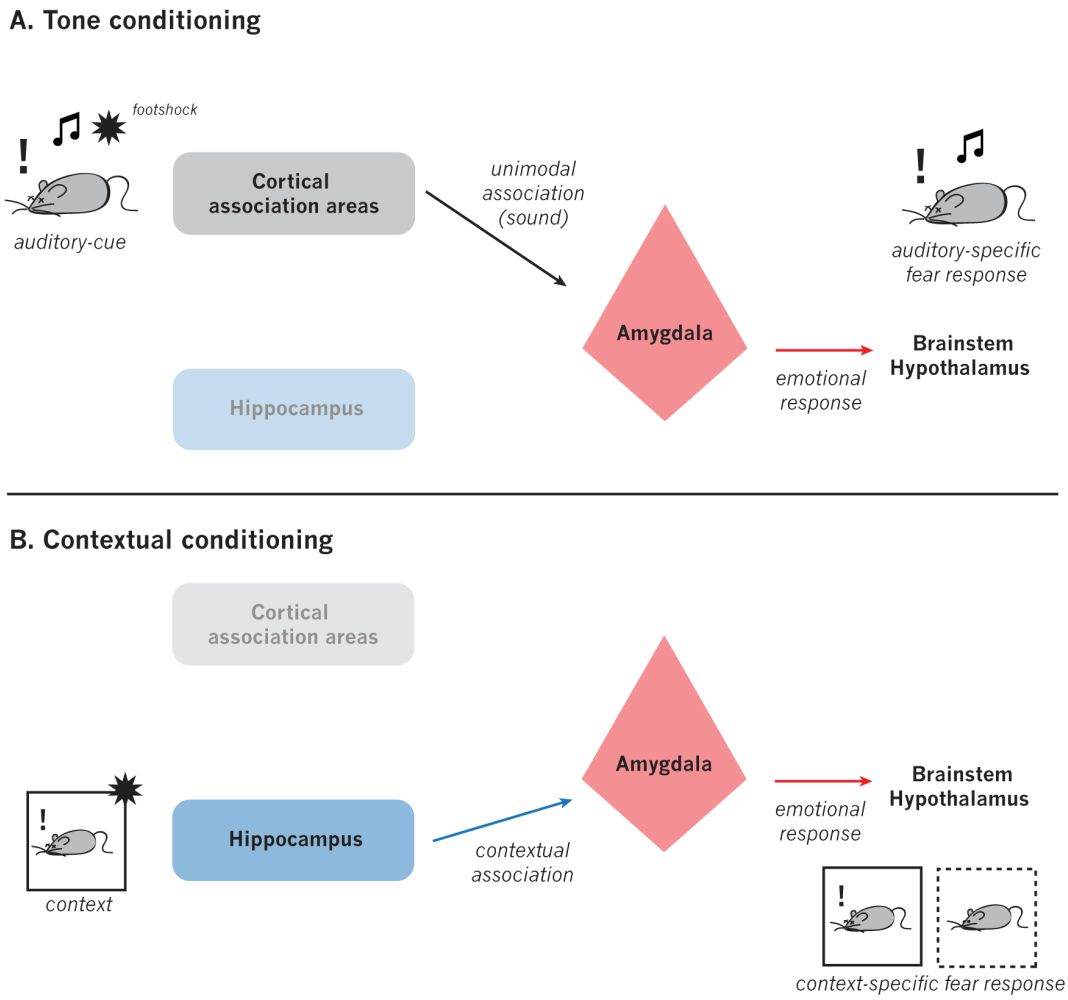

Fig. 1.3: Fear conditioning circuit.

The emotional memory system requires the participation of the amygdala, a group of nuclei located in the temporal lobes of the mammalian brain. **A.** Unimodal information (like for instance, a sound) is received by the lateral nucleus of the amygdala from cortical superior areas (black arrow) and associated to an aversive unconditioned stimulus (a footshock), to create a fear memory. **B.** If the input is contextual, information is received from the hippocampus (blue arrow) by the baso-lateral nucleus, where the association is made. In both cases, the central nucleus of the amygdala mediates the expression of the emotional response through connections to subcortical areas (red arrow) that produce the physiological emotional response (stress hormones release, sympathetic activation, motor behaviour, etc.; Davis, 1992; Kim and Jung, 2006). Scheme inspired by LeDoux (2000) and Squire et al. (2012).

Examples of interactions between memory systems: navigation

Further evidence of parallel processing by memory systems comes from tasks that can be learnt as declarative or procedural memories, such as **navigation** (Chersi and Burgess, 2015; Poldrack and Packard, 2003). Navigation is an essential animal behaviour defined as the ability of organisms to find their way through the environment without getting lost, and depends on several cognitive systems (Geva-Sagiv et al., 2015; Lester et al., 2017). There are basically two types of navigation: **allocentric** (i.e., spatial or place, learnt by the declarative memory system) and **egocentric** (or response, learnt as habits by the procedural memory system). Allocentric navigation uses distal cues and landmarks external to the organism to create a brain representation of a spatial cognitive map. Egocentric navigation follows internal, self-motion and proximal cues and is based on the repetition of a learnt sequence of actions (**Fig. 1.4**; see Chersi and Burgess, 2015).

There is a whole battery of behavioural tests based on maze solving designed to study navigation in animal models and humans, such as the Morris water maze, the plus-maze or virtual reality mazes (Hartley et al., 2003; Vorhees and Williams, 2014). Experiments on these tests have revealed the central role of the hippocampus and the striatum in allocentric and egocentric learning respectively (Hartley et al., 2003; Iaria et al., 2003; McDonald and White, 1994; Morris et al., 1982, 1986; Packard and McGaugh, 1996, 1992). The current view is that the striatum supports stimulus-response associations leading to the formation of rewarded habits, while the hippocampus participates in the representation of spatial relationships in the environment to create the brain cognitive map (Chersi and Burgess, 2015; McDonald and White, 1993; O'Keefe and Nadel, 1978). Noteworthy, hippocampal learning allows i) the update of the memorized information, important in reversal paradigms where the exit of the maze is changed and ii) the flexible use of this information, which is important in novel

situations as when the starting position in the maze is changed (Eichenbaum et al., 1990).

A common example to illustrate the differences between these two strategies is to imagine where you last parked your car. Egocentric, non-spatial navigation would consist on following motion-based rules, such as "go straight ahead and turn right on the second street". This is useful if your car is always parked at the same spot. Allocentric, spatial navigation would consist on marking the position of your car in a cognitive map of your neighbourhood, allowing the flexible update of this map in case you park your car in different streets every day.

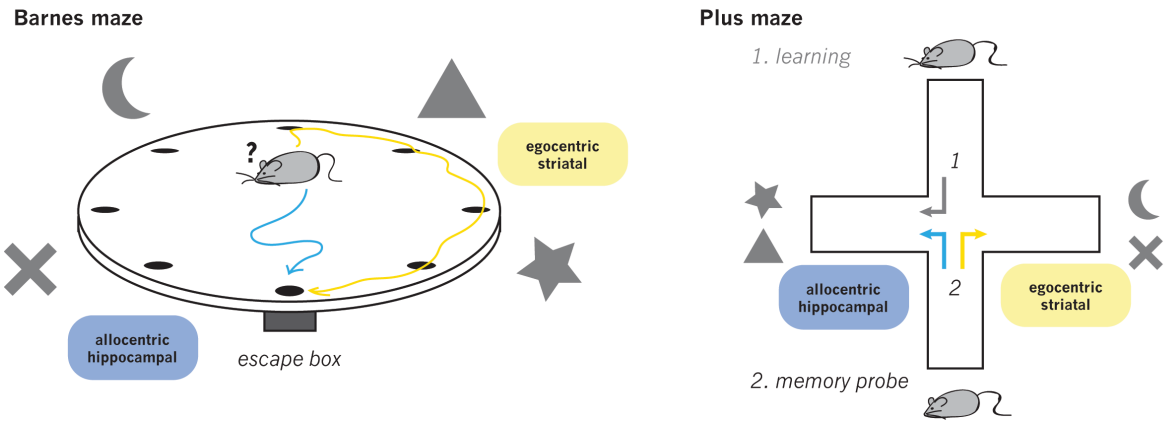

Fig. 1.4: Allocentric vs. egocentric strategies in the Barnes and plus mazes.
Allocentric navigation (blue), based on external cues, relies on the hippocampal construction of a cognitive map representing the environment, where the animal situates itself, reference distal landmarks and the position of the goal. Egocentric navigation consists of the repetition of learnt-sequences of movements. This is illustrated by the use of spatially-directed vs. sequential navigation in the Barnes and plus mazes (Barnes, 1979; Tolman et al., 1946, 1947). Interestingly, distinction between habit and conscious memory were first proposed by philosopher and psychologist William James (1890), who also introduced the distinction between STM and LTM and the notion that memory recall is based on the recollection of organized sets of associations.

Packard and McGaugh (1996) showed that rats learning the plus-maze could use allocentric or egocentric strategies as a function of time and repetition, demonstrating interaction between both memory systems. During a first learning

phase, rats were delivered in a fixed position of the maze, being one of the lateral arms rewarded. Next, rats were tested for memory (probe test) and placed in the opposite starting arm. Specifically, rats probe-tested soon after learning showed allocentric, spatially directed strategies, while rats probe-tested after extended periods of training, when the habit of turning on one direction was consolidated, shifted to egocentric responses (**Fig. 1.4**). Furthermore, they showed that local inactivation of the hippocampus or the striatum led to the expression of the non-inhibited behaviour, demonstrating that both the spatial and striatal representations of the memory exist in parallel and can direct performance when the other system is inhibited (for an example in the water maze, see also McDonald and White, 1994). Alternatively, infusions of glutamate in the hippocampus after training can strengthen the place representation and delay the transfer to the procedural system (Packard, 1999). This interaction between systems can also be competitive, as shown on experiments in which hippocampal lesions facilitated the acquisition of striatal-dependent tasks (McDonald and White, 1993, 1994; Packard et al., 1989) probably by removal of spatial processing that might interfere with the real goal in a sequence-response test (O'Keefe and Nadel, 1978; Poldrack and Packard, 2003).

How these two systems interact to drive performance is still not well understood. On one hand, it has been proposed that direct connections between the striatum and the medial-temporal lobe can competitively recruit these systems (Poldrack and Packard, 2003). Another possibility suggests the participation of a third structure involved in decision making, the medial prefrontal cortex, that could process and compare the output of both memory systems and decide the most appropriate for the task at hand (Chersi and Burgess, 2015).

1.2 THE HIPPOCAMPUS IN THE DECLARATIVE MEMORY SYSTEM

So far, the hippocampus has been highlighted as a key player in the process of memory formation, has been mentioned in several moments and linked to different functions: the effects on episodic memory after the temporal lobes-resection in H.M, the encoding and delivery of contextual information to the amygdala for conditioning or its key function in navigation. With this, I tried to replicate the historical trajectory of hippocampal research, and possibly the reader of this thesis will now be as puzzled about the role of the hippocampus as neuroscience researchers at the end of the 20th century. In the next section, our understanding about the function and structure of the hippocampus will be summarized.

The function of the hippocampus

The mammalian hippocampus (named after the Greek word for sea horse) is the most studied region in the brain. The same way as a neural "Rosetta Stone", deciphering of its structure and function helped in the understanding of many core principles in neuroscience, from the molecular to the cognitive field (Andersen et al., 2009). Many of the hippocampal structural connections and subregions were finely described by Ramon y Cajal (1893). Early believed to be an associative centre serving olfaction, it was not until "A proposed mechanism of emotion", by James Papez (1937), that the hippocampal formation started to be associated to superior cognitive functions, as a key player on the construction of the emotive process. Later on, selective memory impairments in amnesic patients such as H.M. highlighted the role of the hippocampal structures in memory function. The hippocampus has also been broadly used to study the electrical properties of neural tissue, thanks to the laminar disposition of its components and pathways that facilitate the conservation of the circuit in slice

preparations (Schwartzkroin, 1975). This resulted in the discovery of LTP in hippocampal synapses after high-frequency stimulations (Bliss and Lømo, 1973), starting a new era in the molecular and electrophysiological study of memory formation (reviewed in Kandel et al., 2014; Nicoll, 2017). Nowadays, it is accepted that the hippocampus is a central component of the declarative memory system that allows the construction of conscious memories (**Fig. 1.5**).

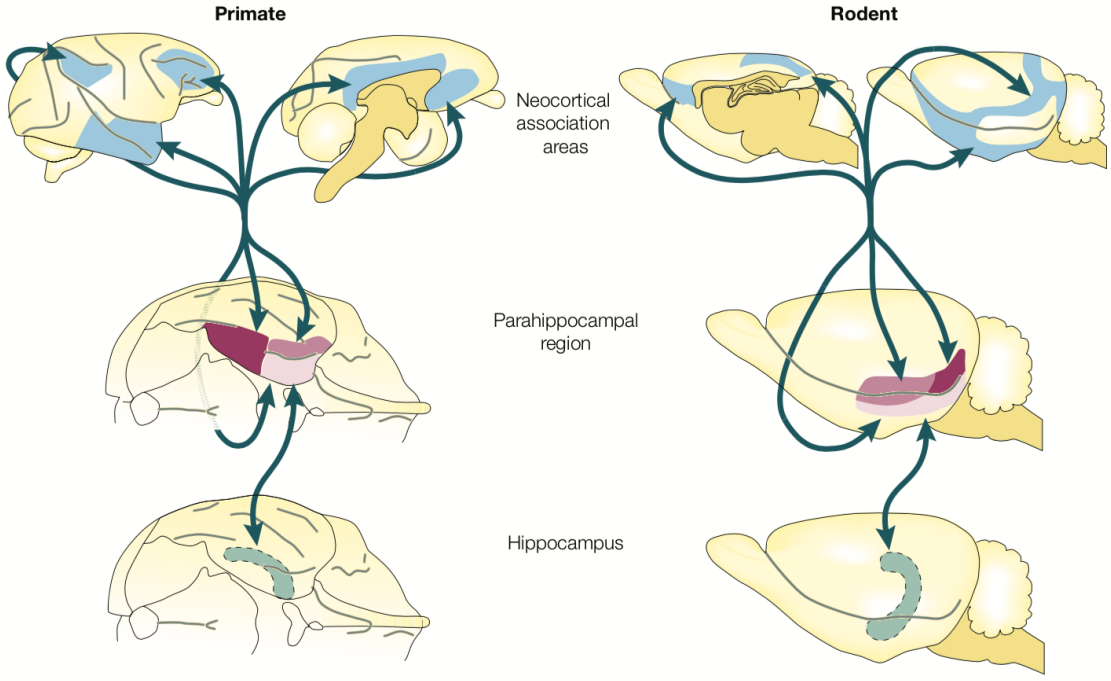

Fig. 1.5: The declarative memory system (extracted from Eichenbaum, 2000).

Multiple associative neocortical areas process highly elaborated information and project to the parahippocampal region, a subdivision of the cortex surrounding the hippocampus that seems to extend the persistence of cortical representations. Once in the hippocampus, information is processed, associated and finally sent back to the same neocortical areas through the parahippocampal cortices. Interestingly, this flow of information defining the hippocampal memory system is very similar in the primate and rodent brain (Burwell et al., 1995).

Among the many theories proposed to explain the role of the hippocampus in memory (for a detailed overview, see Andersen et al., 2009 or Bird and Burgess, 2008), two major views have dominated research on hippocampal function over the last 40 years. The first, called the "*Declarative Theory*", emerged after the

evidence from amnesic patients' inability to recall and generate declarative memories. This theory states that the hippocampus, in conjunction with temporal lobe structures, is fundamental for the brain representations of the relations between objects and events that generate all forms of conscious memories, semantic and episodic (Cohen et al., 1997; Eichenbaum, 2000; Squire, 2004, 1986; Squire et al., 2004). A number of variations of this theory aimed to explain the relative differences in retrieval seen in amnesic patients and animal models. Among these, the "*Index Theory*" elegantly pictures the hippocampus as a librarian that generates indexes of the neocortical patterns active during a given experience, storing the location of the episodic memory and facilitating its recovery (Teyler and DiScenna, 1986; Teyler and Rudy, 2007).

The other major view on hippocampal function is based on navigation experiments, and is summarized in the "*Cognitive-Map Theory*" (O'Keefe and Nadel, 1978). This theory proposes that the hippocampus represents a cognitive map storing allocentric representations to aid flexible, spatial navigation. Although it originally emerged from observations describing the presence of hippocampal "place cells" (neurons that selectively fire in determined locations of the animal in a maze, generating a brain map; O'Keefe and Dostrovsky, 1971), it has now been refined with the discovery of a larger set of spatially-tuned cells supporting the spatial representation view of the hippocampal circuit (Moser et al., 2017; Wills et al., 2014). Both views about hippocampal function were compared and confronted by Eichenbaum and Cohen (2014), who suggested that rather than a selective role in spatial navigation the hippocampus is essential in the relational organization and flexible use of cognitive maps, in which also non-spatial information is processed (Eichenbaum, 2017a; Rubin et al., 2014). In other words, the hippocampus would be dedicated to the mapping, relation and organization of memories in abstract cognitive representations; the "librarian" indexing maps of not only space, but of cognitive space as well (Teyler and Rudy,

2007). These hippocampal representations could be used by many systems in the service of explicit memory recall, navigation, future imagining... (Rubin et al., 2014). Eichenbaum would summarize this idea in the title of one of his last works before passing away: "*the role of the hippocampus in navigation is memory*" (Eichenbaum, 2017b). Different viewpoints on this topic from these and other influential researchers were summarized by Lisman et al. (2017).

Finally, memory is not only about the past but it has also been linked to future planning and imagination, and several experiments are investigating the role of the episodic memory systems and in particular the hippocampus in the simulation of future events and mental time travel (Addis and Schacter, 2008; Irish et al., 2012; Schacter and Addis, 2007).

The basic structure of the hippocampus: the trisynaptic circuit

The study of hippocampal structure, connectivity and neurophysiology is essential in order to understand its role in cognition. The hippocampal formation (often referred to as *hippocampus* for simplicity) includes the dentate gyrus (DG), *cornu ammonis* (Ammon's horn or CA, hippocampus proper) and subiculum, with largely unidirectional connections and three main cortical layers (Witter, 2012).

The hippocampus is derived from the developing cortical *hem* and consists of a folded cortex whose sub-regions range from the subiculum in the distal pole, to the DG in the proximal pole. In rodents, the hippocampal formation presents an elongated banana-shape morphology that extends from its dorsal part in the proximity of the *septum* to its ventral portion in the temporal lobe (septo-temporal axis, longitudinal to the brain; **Fig. 1.5**). All the hippocampal subregions

are present in the septo-temporal axis, such that a transversal section of this axis contains all the hippocampal areas (Andersen et al., 2009; Witter, 2012). In primates the hippocampal formation is not so curved, and due to the expansion of the temporal cortices it lies horizontally in the medial-temporal horn (**Fig. 1.5**; Andersen et al., 2009).

The hippocampus is bridged to multiple association brain areas through the parahippocampal region (entorhinal, perirhinal and postrhinal cortices), where information converges and is re-distributed in a permanent cortico-hippocampal conversation (Eichenbaum, 2000; see **Fig. 1.5**). This way, the hippocampus lies in an ideal position to receive highly elaborated information and encode rapid associations between aspects of experienced events (Henke, 2010).

The entorhinal cortex (EC) and the hippocampus are connected via the trisynaptic circuit (**Fig. 1.6**; Andersen et al., 1971). The EC is considered the first component of this loop and main input to the DG (Ramón y Cajal, 1893; Witter, 2012), and is generally divided in a medial and lateral part. The medial EC, where the famous grid cells are located (Hafting et al., 2005), provide spatial and temporal information about the environment, while the lateral part informs about non-spatial sensory input and object novelty (in other words, contextual information about *where*, *when* and *what* is happening; Hargreaves et al., 2005; Lipton and Eichenbaum, 2008; Robinson et al., 2017; Schlesiger et al., 2015). This information reaches the DG through the perforant path in a topographic manner (Amaral and Witter, 1989; Ramón y Cajal, 1893).

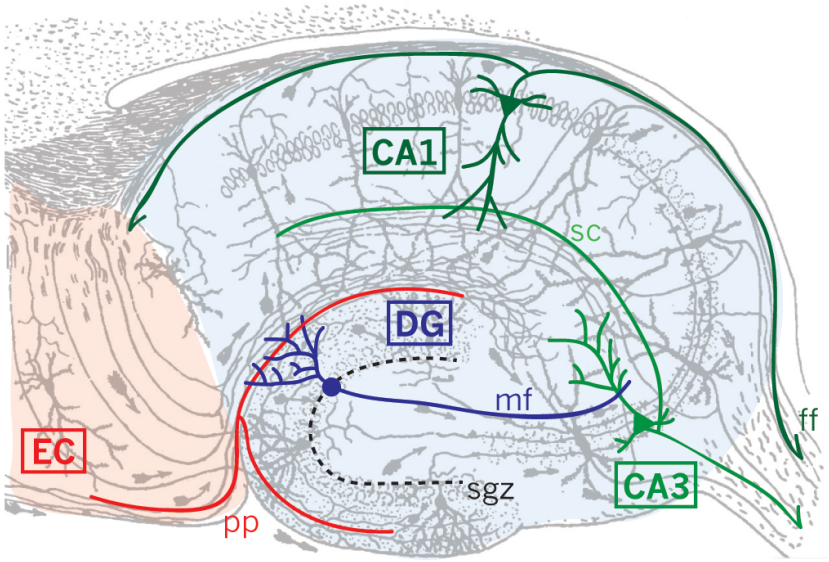

Fig. 1.6: The hippocampal structure (modified from Ramón y Cajal et al. (1909) and Strange et al. (2014). The entorhinal cortex (EC, in red) is connected to the hippocampus (in blue) through the trysinaptic circuit, including the connections: EC → DG → CA3 → CA1. Later on, the information is sent back to the EC and other areas (Andersen et al., 2009; Witter, 2012). In the figure, the circuit is superimposed onto an original drawing from Santiago Ramon y Cajal. pp = performant path; sgz = subgranular zone; mf = mossy fibers; sc = schaffer collaterals; ff = fimbria/fornix fibers.

The main cells of the DG, the granule cells, are organized in a dense V-shaped cell granular layer (GCL) divided in an "upper blade", or suprapyramidal (closer to the overarching CA1) and a "lower blade" or infrapyramidal (opposite to the first one). The granule cells extend their dendrites towards the molecular layer (ML), where the synapsis with the EC inputs take place. Their axons, called mossy fibers, spread exclusively towards the CA3 region through the plexiform layer or hilus (Andersen et al., 2009; Ramón y Cajal, 1893). Laying between the GCL and the hilus, the subgranular zone (SGZ) represents the location of one of the only two adult neurogenic niches in the mammalian brain (Aimone et al., 2014), as it will be further described below. The projection from the EC to the DG is essentially divergent, with the EC sending cortical inputs to the DG in a ratio of 1:10 (Amaral et al., 1990). This, together with the sparse activation of the DG (Chawla et al., 2005; Jung and McNaughton, 1993), amplifies the differences in contextual information encoded by the EC (a computational process

called *pattern separation*; O'Reilly and McClelland, 1994; Treves et al., 2008). In other words, the DG can see contextual differences for which the EC is essentially blind (Valero, 2017), facilitating the non-overlapping representation of contextual information.

On their way to CA3, the mossy fibers contact with hilar interneurons and eventually with the dendrites of CA3 pyramidal cells, forming the *thorny excrescences* (Amaral and Witter, 1989; Ribak et al., 1985). Contrarily to what happened before, the connection between the DG and CA3 is strongly convergent (ca. 10:1; Amaral et al., 1990). This, together with the numerous recurrent connections of the pyramidal cells, make CA3 an optimal *pattern completion* network (the ability to retrieve coherent representations from bits of information; Guzman et al., 2016; Guzowski et al., 2004; Neunuebel and Knierim, 2014). These two processes characteristic of associative networks like the hippocampus have long been thought to be necessary for the optimal storage of information with minimal interference, or in simpler words, keep memories separated while being able to retrieve them from partial cues (McNaughton and Morris, 1987; O'Reilly and McClelland, 1994);
Fig. 1.7).

The next step in the trysinaptic circuit is CA1, whose pyramidal cells receive major inputs from CA3 through the *schaffer collaterals* (Schaffer, 1892), the most studied neural projection in the brain (Andersen et al., 2009). From this region, the connectivity pattern becomes more elaborated, with projections to the subiculum, the EC and subcortical areas like the amygdala, constituting together with the subicular region the main hippocampal output to the brain (Andersen et al., 2009; Witter, 2012). In the CA1 region is where *place cells* were originally described by O'Keefe and Dostrovsky (1971), leading to the hypothesis of the hippocampus serving as a cognitive map for the brain.

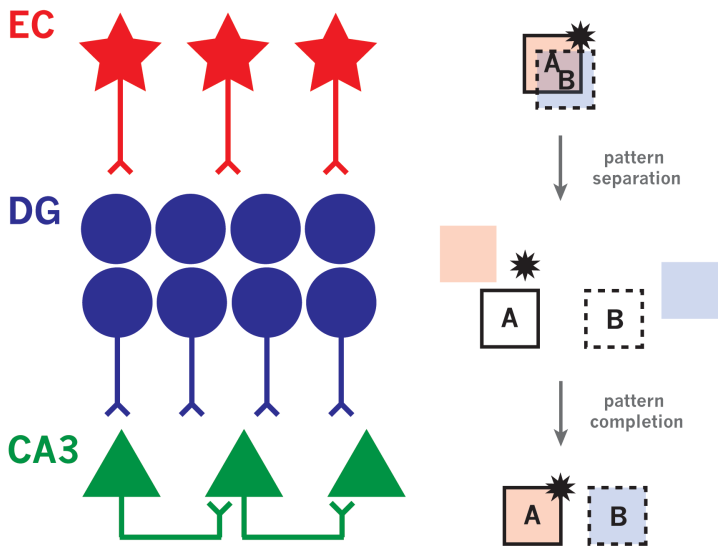

Fig. 1.7: Pattern separation and pattern completion in the hippocampal circuit (inspired by Valero, 2017). Balance between discrimination and generalization of contextual information is believed to be mediated by these two processes, ensuring successful memory storage. The hippocampal network is provided with the neuroanatomy necessary to perform these computations. The divergent projection between the EC and the DG expands the representational space, helping to the separation of overlapping inputs. The convergent connection between the DG and CA3 together with the auto-associative nature of the CA3 circuitry allows the system to reconstruct a memory from partial sets of inputs. (Johnston et al., 2016; O'Reilly and McClelland, 1994; Papp et al., 2007; Treves et al., 2008).

1.3 NEW NEURONS IN THE ADULT HIPPOCAMPUS

Adult hippocampal neurogenesis

The discovery of adult hippocampal neurogenesis in the rodent DG by Joseph Altman and Gopal Das (1965) challenged the long-term assumption that the adult brain was incapable of making new neurons, and opened the research field of memory to a new level of structural plasticity. Ever since, hippocampal neurogenesis has been documented in a number of species including humans (Boldrini et al., 2018; Eriksson et al., 1998; Spalding et al., 2013), and a broad literature helped in the understanding of this process and its potential relevance for the hippocampal function and cognition (for a comprehensive read, see Kempermann, 2011).

Given the fact that neurons are mostly post-mitotic, Altman's findings suggested the presence of precursor cells in the SGZ (a term coined also by him in a later study; Altman and Bayer, 1975). These cells were first described *in vitro* by Palmer et al. (1997, 1995) and later on *in vivo* by Seri et al. (2001). Now, we know that an endogenous and heterogeneous population of neural stem cells (NSCs) resides in the hippocampal SGZ and gives rise to new granule neurons that integrate in the hippocampal DG circuit throughout life (Aimone et al., 2014; Bonaguidi et al., 2012; Bond et al., 2015; **Fig. 1.8**).

The primary NSC population in the SGZ seems to be formed by a group of cells with passive membrane properties, triangular soma morphology, radial process and glial markers like nestin, GFAP and Sox2 (Filippov et al., 2003; Kempermann et al., 2004; Kronenberg et al., 2003; Seri et al., 2001; Steiner et al., 2006). These radial glia-like NSCs, also called type 1 cells, are abundant and mostly quiescent,

as shown in proliferation experiments performed with thymidine analogues like BrdU (Filippov et al., 2003; Kronenberg et al., 2003). Interestingly, the existence of a non-radial primary NSC has also been reported (Suh et al., 2007), emphasising the heterogeneity of the NSC pool (Bonaguidi et al., 2012; Lugert et al., 2010). The entry into the cell-cycle is a process highly regulated by different morphogenetic mechanisms (Han et al., 2015; Lie et al., 2005; Mira et al., 2010), neurochemical signalling (Crowther and Song, 2014; Song et al., 2012; Zaben Malik et al., 2009) and the animal's experience (Dranovsky et al., 2011). Upon activation, the main mode of division at the population level is asymmetric followed by terminal astrocytic differentiation, exhausting the NSC pool with time (Encinas et al., 2011; Encinas and Sierra, 2012), although symmetric divisions have also been reported in single clones (Bonaguidi et al., 2011).

Asymmetric divisions generate fast-proliferating intermediate progenitors (IPs) that rapidly amplify before differentiation (Kempermann et al., 2004). These cells (type 2) are horizontally oriented, retain Sox2 and nestin expression (type 2a) and start showing neuronal markers like NeuroD or doublecortin (DCX, type 2b; Brown et al., 2003; Kronenberg et al., 2003; Steiner et al., 2006). More committed neuroblasts or type 3 DCX+ cells transiently express the Ca^{2+}- binding protein calretinin, have lower proliferative potential and variable morphologies, in accordance to their maturational state (Brandt et al., 2003; Brown et al., 2003). When these neuroblasts exit the cell-cycle they stop expressing calretinin and DCX, now showing calbindin and NeuN immunoreactivity. As they migrate into the thickness of the GCL, newborn neurons are recruited into the hippocampal circuitry in an activity-dependent manner that determines their survival (Biebl et al., 2000; Kempermann et al., 2003; Sierra et al., 2010; Tashiro et al., 2006). Four weeks after birth, newborn neurons express mature markers and show elaborated dendritic arbours coated with dendritic spines and functional connections with CA3 (Toni et al., 2008; van Praag et al., 2002; Zhao et al., 2006).

Seven to eight weeks after birth, newborn neurons become indistinguishable from the developmentally generated granule cells (Aimone et al., 2014; Zhao et al., 2006).

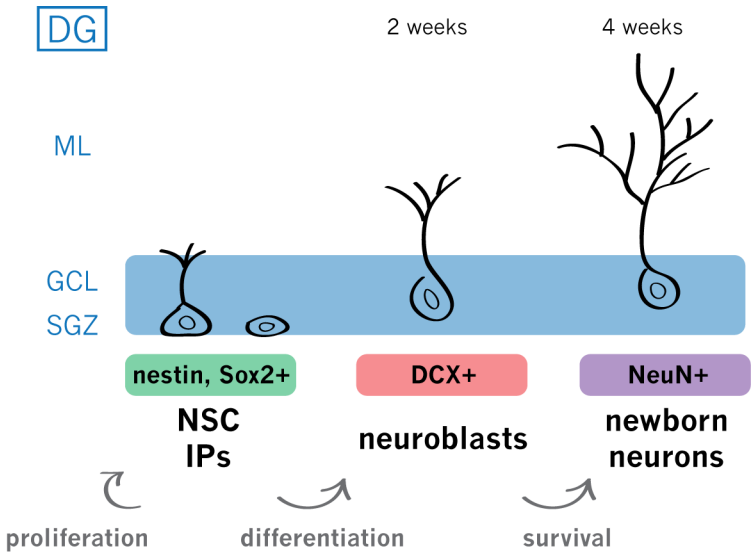

Fig. 1.8: Adult hippocampal neurogenesis.

Summarized developmental stages of adult hippocampal neurogenesis, with the most important steps and markers relevant for the understanding of this thesis. Noteworthy, adult neurogenesis also occurs in the lateral wall of the cerebral ventricles, in a region named subventricular zone (SVZ) providing neurons to the olfactory bulbs. In the interest of simplicity, this introduction is mainly focused in the process of adult hippocampal neurogenesis.

Regulation of adult hippocampal neurogenesis

Neurogenesis is a very dynamic process. Possibly one of its most interesting features is its sensitivity to regulation, ultimately controlling the generation and/or integration of newborn neurons. There is an extensive literature reviewing a number of internal and external factors influencing neurogenesis at different levels (Aimone et al., 2014; Kempermann, 2011; **Fig. 1.9**). From a functional perspective, the production of more or less neurons is thought to respond to the cognitive needs of the individual, to cope with high or low circuit processing requirements (Kempermann, 2008). Intuitively, exposing the animals to

cognitively complex and novel environments has positive effects on neurogenesis. Two of the most used paradigms modelling such stimulation are physical exercise and environmental enrichment, facilitating proliferation and survival of newborn neurons respectively (Kempermann et al., 1997; Henriette van Praag et al., 1999). Even the process of learning itself promotes neurogenesis (Epp et al., 2007; Gould et al., 1999; Petsophonsakul et al., 2017; Zhao et al., 2006). Alternatively, detrimental physiological factors like stress or aging (about which I will further elaborate below) can negatively influence neurogenesis as well (Dranovsky et al., 2011; Gould et al., 1997).

How the neurogenic niche senses these external changes and the search of their molecular counterparts are object of intense research. At the circuit level, hilar GABA interneurons have been proposed to respond to neuronal activity in the DG and control NSC activation and neuroblast survival according to experience (Dranovsky et al., 2011; Song et al., 2012, 2013). At the systemic level, a plethora of factors acting on neurogenesis and released in different conditions such as physical exercise or stress has been identified (reviewed in Aimone et al., 2014; Kempermann, 2011).

It was early noticed that, the same way as environmental factors have an effect in neurogenesis levels, the number of newborn neurons can also affect behaviour, in particular learning and memory (Shors et al., 2002; Snyder et al., 2005; van Praag et al., 1999). In fact, much of what we know about neurogenesis cognitive role comes from experiments that exploit its high responsiveness to regulation. Most of the available gain of function models, functionally more attractive because of its regenerative potential, have strong secondary systemic effects (running and enriched environment; reviewed in van Praag, 2009; van Praag et al., 2000), hampering the interpretation on the specific share of neurogenesis in cognitive improvements. To circumvent this problem more selective, conditional genetic

models were developed, although many of them increase neurogenesis by interfering with physiological processes. For instance, the Bax knock-out model increases neurogenesis by preventing a cell death program that is thought to remove superfluous and unnecessary neurons from the circuit, artificially modifying the balance between immature and mature neurons (Sahay et al., 2011a; Sun et al., 2004). More newborn neurons competing for the same synaptic afferents from the EC interferes with the integrity of the previously established circuit (Adlaf et al., 2017; Ryu et al., 2016), which might impair pattern completion (Nakashiba et al., 2012). Something similar occurs with the Klf-9 knock out model (McAvoy et al., 2016), in which synapses from developmentally generated granule cells are removed to favour NSC proliferation and newborn neurons integration. Therefore, more specific models are needed to unravel the specific impact of a physiological increase in neurogenesis on hippocampal function.

Previous studies in the lab where this thesis was conducted developed a new method that could extend our knowledge about the neurogenic process and its functional implications. Briefly, this technique was established after the observation that the manipulation of the G1 phase of the cell-cycle changes the balance between proliferation and differentiation of NSCs (Calegari et al., 2005; Lange et al., 2009). Such idea was successfully used to study corticogenesis, where the overexpression of two positive cell-cycle regulators (cdk4 and cyclinD1, from now on referred to as 4D) expanded the population of basal progenitors and resulted in increased surface and gyrification of the mouse and ferret brains, respectively (Nonaka-Kinoshita et al., 2013). Translation of this method into the adult brain allowed an *in vivo* controlled expansion of the endogenous pool of NSCs and posterior increase in neurogenesis in the adult DG and SVZ (Artegiani et al., 2011; Bragado-Alonso et al., *submitted*). Yet, the potential suitability of

this model for the study of neurogenic contribution to cognition has not been addressed.

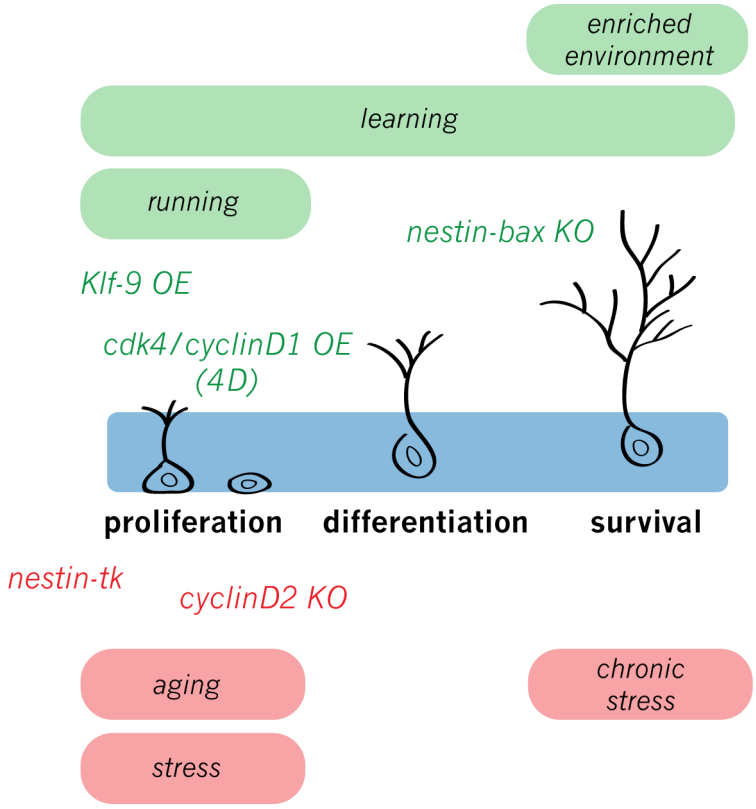

Fig. 1.9: Regulation of adult hippocampal neurogenesis (inspired by Aimone et al., 2014).
Positive (green) and negative (red) external factors (inside the boxes), as well as more specific, genetic models (outside the boxes) controlling neurogenesis at different stages (reviewed in Aimone et al., 2014; Kempermann, 2011). OE = overexpression; tk = thymidine kinase; KO = knock-out.

The function of adult hippocampal neurogenesis

Finding the role of newborn neurons in the hippocampal system has been object of extensive research and the ultimate question in the adult neurogenesis field. Importantly, it has been proposed that its presence in the DG is far from being a mere atavism of the brain, but rather represents an actively conserved evolutionary trait essential for DG function and adaptability (Kempermann,

2016). Being neurogenesis as it is an extraordinary level of plasticity in the DG, newborn neurons could be supporting the function of this structure on pattern separation processing and promote cognitive flexibility (Aimone et al., 2014; Anacker and Hen, 2017; Besnard and Sahay, 2016; Johnston et al., 2016; Nakashiba et al., 2012; Sahay et al., 2011b). It is important to remember that adult-generated cells do not act in isolation but as part of an elaborated hippocampal circuit (Vivar and van Praag, 2013). However, it is remarkable that several experiments revealed differential contributions of newborn neurons to learning and memory in spite of their small numbers (around 0.05% and 0.004% neuronal exchange rate per day in rodents and humans respectively; Imayoshi et al., 2008; Spalding et al., 2013).

As an example, several loss-of-function approaches blocking neurogenesis have found impairments in tasks demanding disambiguation of highly similar information (i.e., pattern separation), like allocentric navigation paradigms including reversal as well as spatial/contextual discrimination (Bekinschtein et al., 2014; Burghardt et al., 2012; Clelland et al., 2009; Dupret et al., 2008; Garthe et al., 2009, 2014; Guo et al., 2011; Nakashiba et al., 2012; Niibori et al., 2012; Pan et al., 2012; Swan et al., 2014; Tronel et al., 2012; Wojtowicz et al., 2008; Wu and Hen, 2014; Zhang et al., 2014). Conversely, gain-of-function experiments have shown increased flexible learning and pattern separation in similar hippocampal-dependent tasks (Garthe et al., 2016; Kent et al., 2015; McAvoy et al., 2016; Sahay et al., 2011a; Wang et al., 2014), in line with early theories involving neurogenesis in the reduction of memory interference (Wiskott et al., 2006). As a consequence, those memories are more precise and their neural representations are kept separated, allowing cognitive flexibility and reducing generalization over time (Aimone et al., 2011; Anacker and Hen, 2017; Besnard and Sahay, 2016; McAvoy et al., 2015; **Fig. 1.10**). Interestingly, neurogenesis can also reduce interference by facilitating forgetting of conflicting old memories (Epp

et al., 2016) and mediating a rapid transfer of the memory to cortical circuits (Kitamura et al., 2009).

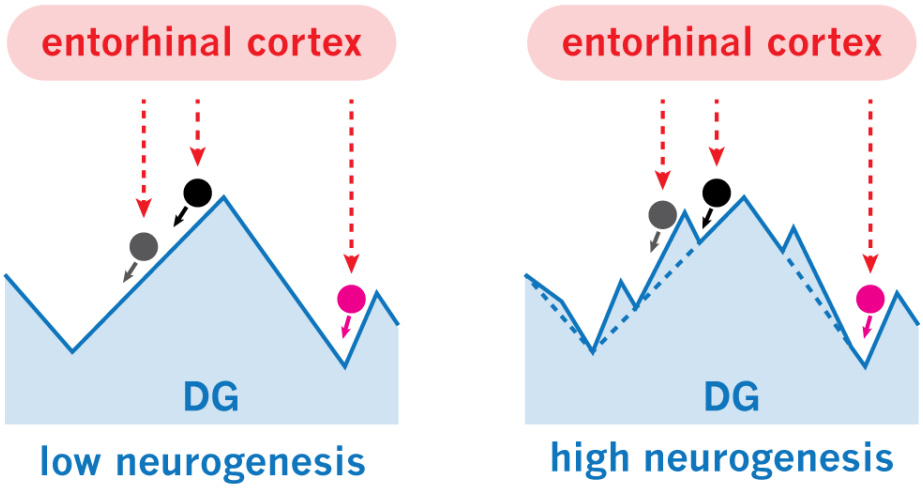

Fig. 1.10: Effect of neurogenesis in pattern separation (inspired by Johnston et al., 2016).
Scheme representing an intuitive way of picturing pattern separation in the DG in conditions of low and high neurogenesis. The DG, depicted as a hilly land-scape, separates the information received by the EC (balls rolling on the hills). Neurogenesis would act by making more different representations of those inputs, increasing the resolution (roughness) of the landscape. In conditions of low neurogenesis, two similar inputs (black and grey balls) would end up in the same valley, still different from a very different input (pink ball). By increasing neurogenesis, the two similar inputs are stored in different representations (valleys). Following a previous example, increasing neurogenesis would allow you to better distinguish between the location of your car yesterday and the day before.

How a few newborn neurons make such a differential contribution to DG function is under debate (Ming and Song, 2011; Piatti et al., 2013). Four-to-six weeks old newborn cells are known to exhibit a critical period of hyperexcitability and increased synaptic plasticity compared to developmentally generated granule neurons, making them efficient substrates of Hebbian potentiation (Espósito et al., 2005; Gu et al., 2012a; Schmidt-Hieber et al., 2004; Snyder et al., 2001). Being highly excitable in a mostly "silent" structure as the DG makes them easier to recruit during learning tasks (Kee et al., 2007). In other words, they are particularly sensitive and responsive to the information reaching the DG (Danielson et al., 2016; Marín-Burgin et al., 2012; Saxe et al., 2006). Newborn

neurons have also been suggested to preferentially reactivate upon stimuli they were exposed to during maturation, (Aimone et al., 2011; Tashiro et al., 2007; Trouche et al., 2009), introducing the idea of a shift from encoding to retrieval functions as they mature. Similarly, their transient period of hyperexcitability could facilitate the association of temporarily related events (Aimone et al., 2006).

Although these findings support the notion that adult-generated neurons act as encoding units, a different view suggests that they mainly dictate DG activity by feed-back inhibition through hilar interneurons (McAvoy et al., 2015; Ming and Song, 2011; Sahay et al., 2011b). It has been shown that increasing neurogenesis silences the DG circuit, an important condition for pattern separation, whereas ablation of the hyperexcitable newborn neurons paradoxically increases the excitability of the DG (Burghardt et al., 2012; Drew et al., 2016; Ikrar et al., 2013) and CA3 (Niibori et al., 2012), what may increase overlapping between neural representations or, in other words, memory interference (Besnard and Sahay, 2016; Burghardt et al., 2012; McAvoy et al., 2015; Niibori et al., 2012). This hypothesis puts neurogenesis in a central position in the government of DG function as a structure, and would help to explain the strong influence of neurogenesis in learning and memory. However, since newborn granule cells have recently been shown to recruit effective feed-back inhibition only at late phases of maturation (Temprana et al., 2015), more studies are needed to fully understand how this structural modulation of DG activity takes place.

Neurogenesis is an oddity. Not only it provides an ideal model to study stem cell biology and regenerative approaches *in vivo*, but also to enhance cognition given its high responsiveness to external regulation and unique position to modulate hippocampal memory function. At this respect, it has been proposed that neurogenesis could constitute a principal component of the neural reserve capacity of the brain to cope with environmental changes over the lifespan (Kempermann,

2008; **Fig. 1.11**). Whether adult neurogenesis can be extrinsically exploited as a brain reserve to preserve cognition throughout life will be the central question of this thesis.

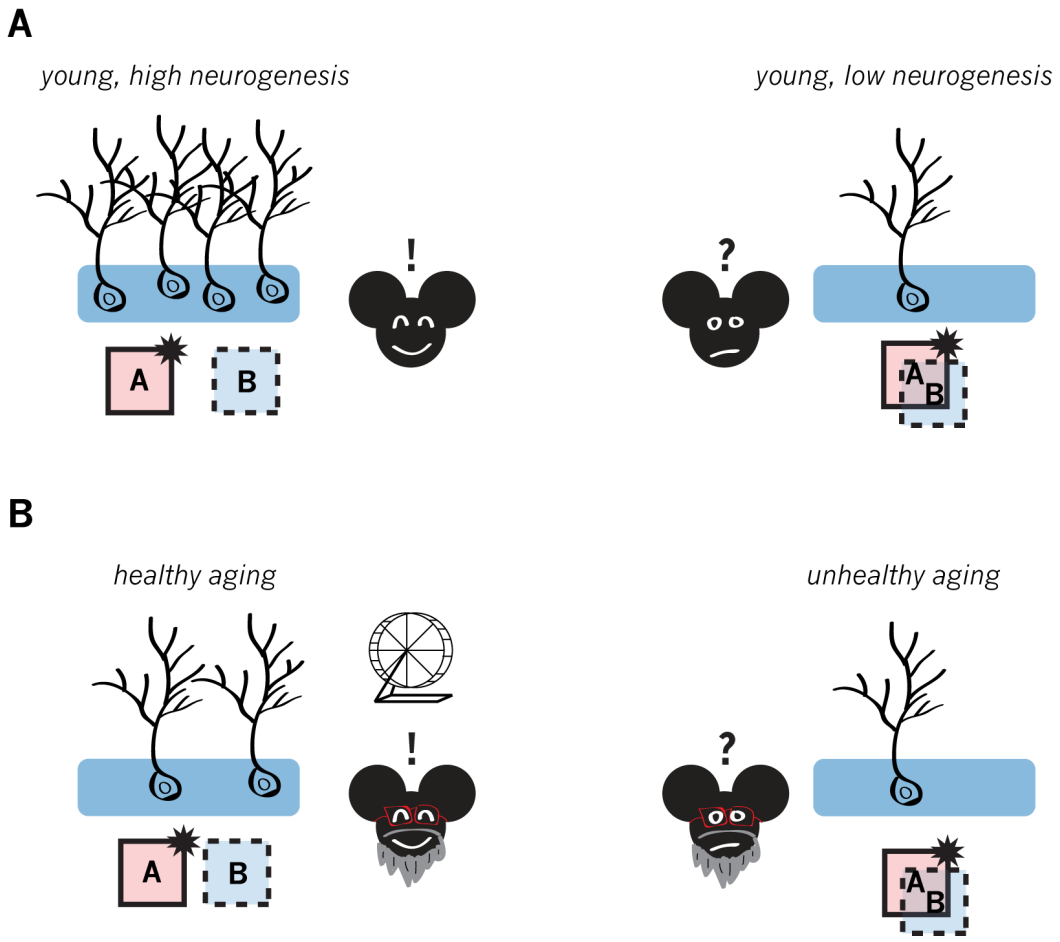

Fig. 1.11: Neurogenic function and reserve hypothesis (inspired by Kempermann, 2008).

A. Different experiments showed the positive effect of increased neurogenesis in pattern separation, depicted as effective discrimination between two similar contexts A and B. From an evolutionary point of view, a better discrimination could help in the adaptability of the animal to new experiences and contribute to survival. Given the high responsiveness of neurogenesis to external stimuli, neurogenesis could act as a sensor of environmental activity and accommodate information processing at the level of the complexity and novelty encountered (an elegant illustration of this idea correlated individual differences in neurogenesis levels to exploratory behaviour in genetically identical mice; Freund et al., 2013). **B.** With physical or cognitive stimulation, neurogenesis could be exploited as a cognitive reserve and contribute to healthy aging.

1.4 THE EFFECT OF AGING IN THE HIPPOCAMPAL MEMORY SYSTEM

Age-related decline in hippocampal function

World's population is aging rapidly. People aged over 60 are predicted to rise over two billion by 2050 (25% of total population), suffering dementia 5% to 7 % of this group of people (Prince et al., 2013). Therefore, the urgent identification of the mechanisms ensuring healthy aging becomes one of the central goals in modern medicine.

Aging has distinctive effects in the brain, being impairments in the declarative memory system more pronounced than in other cognitive functions (Leal and Yassa, 2015; Lester et al., 2017; Samson and Barnes, 2013). This points to a special vulnerability of the medial-temporal lobe structures, particularly the hippocampus, during aging (Hedden and Gabrieli, 2004). Consequently, studies in humans have reported a reduction in hippocampal volume and associated cortices during aging (Raz et al., 2010; Tisserand et al., 2000; Yassa et al., 2010) that correlates with poor cognitive performance (Konishi and Bohbot, 2013; O'Shea et al., 2016; Raz et al., 1998; Shing et al., 2011) and is accentuated in pathological conditions like in Alzheimer Disease (Jack et al., 1999). Yet, the causes of this special vulnerability remain elusive and mechanisms that may compensate for it are not known.

An increased susceptibility to **contextual interference** (e.g. loss of details or difficulties to distinguish among similar experiences) and impairments on **spatial memory** (the ability to navigate in an environment) are among the most important memory deficiencies in the aging brain (Leal and Yassa, 2015; **Fig.**

1.12). Both aged humans and rodents show difficulties in tasks that require discrimination between similar memories (Burke et al., 2010; Gracian et al., 2013; Holden et al., 2012; Reagh et al., 2014; Stark et al., 2013; Wu et al., 2015), indicative of impairments in pattern separation (Holden and Gilbert, 2012). Consistently, experiments have shown less perforant pathway synapses between the EC and the DG, hampering DG potentiation and correlating with memory impairments in aged rodents (Barnes, 1979; Barnes et al., 2000; Smith et al., 2000; also mimicking findings in humans, Yassa et al., 2010). This is in line with a reduced activity of the DG after behaviour (Cleland et al., 2017; Penner et al., 2011) which, together with an hyperexcitable CA3 region (Wilson et al., 2005), could affect the balance between pattern separation/completion in the old DG/CA3 circuit and disturb the encoding and retrieval of contextual memories (Leal and Yassa, 2015; McAvoy and Sahay, 2017). This is also illustrated by other experiments showing failure in memory engram reactivation in the old DG after re-exposition to the same context (Marrone et al., 2011) and impaired consolidation (Oler and Markus, 1998), showing clear deficits in the recovery of contextual memories.

These hippocampal deficits in contextual memory processing, as well as changes in sensory systems and perception, also mediate impairments in spatial navigation (for a review, see Lester et al. (2017)). Early studies in rodents have shown that navigational learning switches during aging from allocentric/place, based on the construction of an hippocampal cognitive map to egocentric/response strategies, based on striatal, self-motion sequences (Bach et al., 1999; Barnes et al., 1980). The same occurs in humans, where shift from place to response strategies correlates with decreased hippocampal vs. striatal activity (Bohbot et al., 2012; Head and Isom, 2010; Konishi and Bohbot, 2013; Kyoko et al., 2013). Apart from the aforementioned deficits in contextual processing that could affect processing of spatial information, specific impairments in the plasticity and specificity of the

old CA1 place cells have been suggested to influence the stability of the cognitive maps used for navigation (Barnes et al., 1997; Lester et al., 2017; Shen et al., 1997). Moreover, the sequential reactivation of these cell assemblies that is thought to mediate consolidation during sleep and rest periods is temporally disrupted and weakened in aged rats (Gerrard et al., 2008; Wiegand et al., 2016).

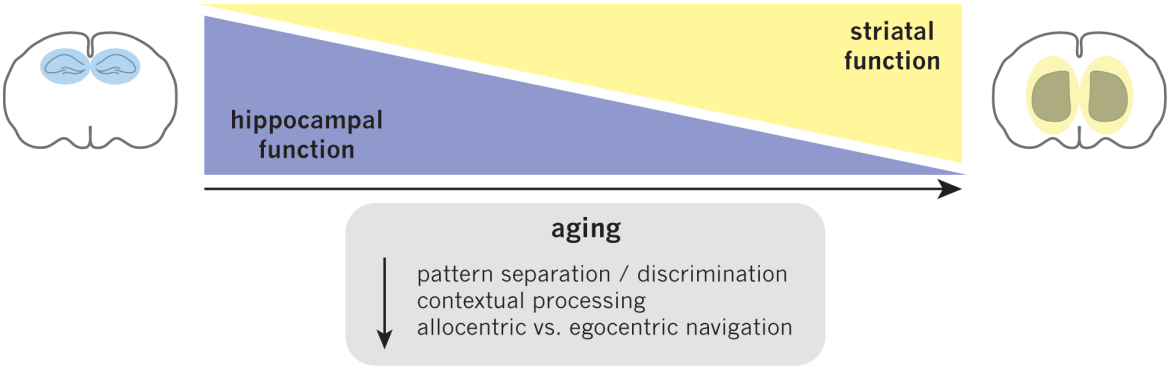

Fig. 1.12: Age-related decay in hippocampal function.
The function of the hippocampus declines with age due to different impairments at the level of neuronal physiology and circuitry (for compehensive reviews further describing age-related changes in hippocampal function, including at the level of neuromodulation, trophic factors, molecular regulation or receptor-mediated plasticity, see: Leal and Yassa, 2015; Lester et al., 2017; Burke and Barnes, 2006). These changes lead to impaired contextual learning and a switch from hippocampal, allocentric to striatal, egocentric navigational strategies in old individuals.

Age-related decline in neurogenesis

Interestingly, the effects of aging in hippocampal plasticity also include a dramatic reduction in the levels of adult neurogenesis (Aimone et al., 2014; Kempermann, 2011; Lee et al., 2012). This was first noticed by early reports assessing proliferation in aged rodents, including the seminal work by Joseph Altman and Gopal Das (Altman and Das, 1965; Cameron and McKay, 1999; Kuhn et al., 1996). Neurogenesis reaches its highest levels early during adulthood and quickly declines thereafter (Ben Abdallah et al., 2010; Morgenstern et al., 2008). This has

led to the hypothesis that aging might be a co-variable affecting many systemic processes (Kempermann, 2011). No major differences have been described in neuronal differentiation or morphology in the aged DG (Couillard-Despres et al., 2006; Morgenstern et al., 2008). Rather, age-related decline in neurogenesis seems to be a consequence of the lower proliferative potential and/or exhaustion of the hippocampal NSC pool (Bonaguidi et al., 2011; Encinas and Sierra, 2012; Hattiangady and Shetty, 2008; Olariu et al., 2007). These intrinsic effects can be influenced by external signals affecting the neurogenic niche where NSCs reside (Seib and Martin-Villalba, 2015). Several growth factors, hormones and morphogens known to affect NSC activity are altered in the aged hippocampus (Shetty et al., 2005), and manipulations preventing these alterations during aging increase neurogenesis (Cameron and McKay, 1999; Licht et al., 2016; Montaron et al., 2006; Seib et al., 2013; Yousef Hanadie et al., 2014). This is of high relevance as it indicates that the old brain keeps some of its neurogenic potential, as also shown in physical activity or enriched environment paradigms (Kempermann et al., 1998; Kronenberg et al., 2006; van Praag et al., 2005). A beautiful compilation on the rejuvenating effect of these and other factors on neurogenesis and cognition was studied in parabiosis experiments, where young and old mice shared their systemic environment (Villeda et al., 2011). Importantly, these studies highlighted the potential of neurogenesis to improve cognition in the old brain (Montaron et al., 2006; Seib et al., 2013; van Praag et al., 2005; Villeda et al., 2011; Wu et al., 2015), although the correlation between neurogenesis levels during aging and cognitive performance is still unclear (Bizon and Gallagher, 2003; Drapeau et al., 2003; Gil-Mohapel et al., 2013).

A recent report, selectively increasing neurogenesis through the modulation of neuronal competition in the DG, also described improvements in contextual memory precision during aging (McAvoy et al., 2016). Detailed analysis in DG activity upon increased neurogenesis revealed more global remapping (i.e., less

overlapping) among granule cell assemblies in memory interference tasks. This study started to fill the gap in the circuitry mechanisms behind the rejuvenating effect of neurogenesis in cognitive function, and reinforced the idea that neurogenesis can be targeted during aging for the improvement of memory circuits (Kempermann, 2008; Lepousez et al., 2015; McAvoy and Sahay, 2017). Complementary studies selectively increasing neurogenesis during aging are needed to corroborate this hypothesis and help in the understanding of how newborn neurons ameliorate age-related cognitive decline.

1.5 AIM OF THE PROJECT

The main goal of this thesis is to help in the understanding of neurogenesis contribution to memory function, during youth and aging, using the 4D model as a reliable method to increase the neurogenic output in the hippocampal circuit.

First, I will comprehensively investigate the consequences of the 4D model in the neurogenic process and the impact of increased neurogenesis on hippocampal function during youth.

Second, I will address the potential benefit of increased neurogenesis across the lifespan, with the goal to ascertain whether neurogenic reserves can be exploited during aging to ameliorate the effects of senescence in brain function.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Bacteria, cell lines and mouse strains

Bacteria, cell or mouse line	Supplier
One Shot Top-10 *E.coli*	Thermo Fisher
293T cells	Gift from the Lindemann lab
C57BL/6JRj	Janvier Labs
nestin::CreERt2	(Imayoshi et al., 2006)

Table 2.1: Bacteria and cell/mouse lines

2.1.2 Plasmids

Plasmid	Source
pczVSV-G (viral envelope)	(Pietschmann et al., 1999)
pCD/NL-BH (gag-pol)	(Mochizuki et al., 1998)
p6nts-GFPloxpNLSloxp	(Artegiani et al., 2011)
p6nts-GFPloxpNLS4Dloxp	(Artegiani et al., 2011)

Table 2.2: Plasmids for viral preparation

2.1.3 Primers used for genotyping

nestin::CreERt2	5' GTTTCACTGGTTATGCGGCG 3'
	5' GAGTTGCTTCAAAAATCCCTTCC 3'

Table 2.3: Primers used for genotyping

2.1.4 Chemicals, buffers and culture media

In general, chemicals were purchased from Invitrogen, Life Technologies, Merck, Roche or Sigma-Aldrich. Standard buffers, buffers used for histology and culture media are showed.

Buffer	Composition
Phosphate buffer (PB)	110 mM Na_2HPO_4/NaH_2PO_4 in H_2O pH = 7.4
Phosphate buffer saline (PBS)	137 mM NaCl 2.7 mM KCl 10 mM Na2HPO4 1.8 mM KH_2PO_4 in H_2O pH = 7.4

Table 2.4: Standard buffers.

Buffer/solution	Composition
Blocking/permeabilization solution	0.3% Triton-X-100 10% Donkey Serum in PBS
Cryoprotectant solution	25% Ethylenglycol 25% Glycerol 50% PBS 2X
DNA denaturalization solution	HCl 2M in H_2O
Fixation buffer	4% Paraformaldehyde in PB pH = 7.4
Incubation buffer	0.3% Triton-X-100 3% Donkey Serum in PBS
Quenching solution	0.1M Glycine in PBS pH = 7.4

Table 2.5: Histology and immunohistochemistry buffers.

Medium	Composition
LB medium (CRTD media kitchen)	10 mg/ml NaCl 10 mg/ml Bacto-tryptone 5 mg/ml Bacto-yeast extract in H_2O
Cell culture medium	DMEM (Gibco) 10% Fetal bovine serum 100 U/ml Penicillin-streptomycin

Table 2.6: Culture media.

2.1.5 Antibodies

Antigen	Dilution	Supplier	Catalog
Sox2	1:100	Santa Cruz	SC-17320
S100ß	1:1000	Abcam	ab14688
GFP	1:500	Thermo Fisher	A-11122
BrdU	1:250	Abcam	ab6326
DCX	1:100	Santa Cruz	SC-8066
NeuN (Fox3)	1:500	Abcam	ab104225
Arc	1:1000	Synaptic systems	156005
c-Fos	1:1000	Synaptic systems	226003

Table 2.7: Primary antibodies.

Secondary antibodies were IgG raised in donkey (against goat, mouse, rabbit, rat and guinea pig), conjugated to different fluorofores (DyLight, Alexa or Cyanines), all purchased from Jackson Immunoresearch.

2.2 METHODS

Viral preparations

Viruses were produced by polyethyleneimine co-transfection of 293T cells with the respective transfer vector (GFP or 4D; **Fig. 2.1**) and plasmids coding for the HIV-1 gag/pol and VSV-G proteins in a proportion 1:1:1, as described by Artegiani et al. (2011, 2012). In particular, 4D viruses encoded for the three transgenes (GFP^{nls}/Cdk4/cyclinD1) linked by 2A peptides and LoxP sites allowing, respectively, their stoichiometric expression and recombination of the 4D cassette together with the nuclear localization signal (NLS) of GFP upon tamoxifen administration in *nestin*::CreRTt2 mice (Artegiani et al., 2011; **Fig. 2.1**). 24 hours after transfection the medium was replaced by serum free medium and 1 day later the filtered supernatants were centrifuged at 25.500 rpm for 3 hours. The viral particles were suspended in 40 µl of PBS per 10 cm petri dish and further concentrated using centrifugal filters (Amicon). This yielded ca. 40 µl of virus suspension per construct with a titer of 10^8-10^9 IU/ml as assessed by titration of HEK cells.

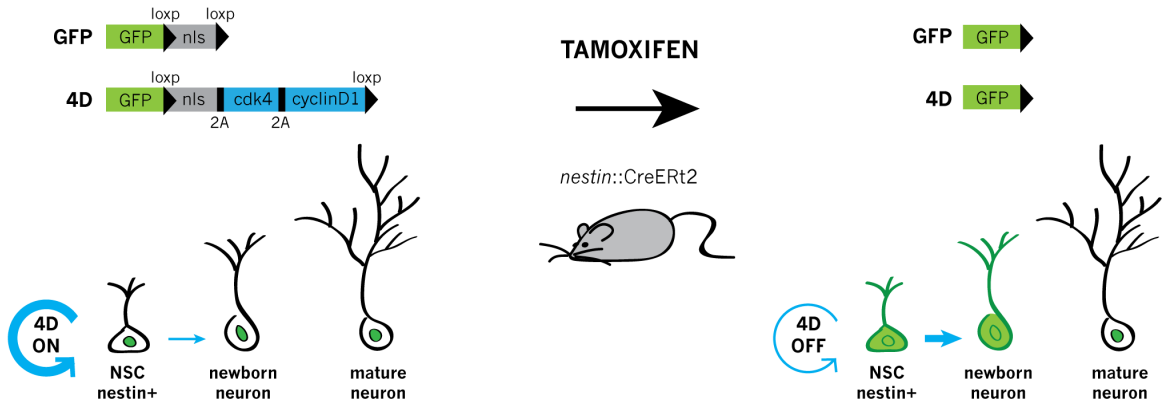

Fig. 2.1: Viral constructs design.

Scheme depicting the GFP and 4D viral constructs. Temporal control of transgenes expression was achieved by injecting *nestin*::CreERt2 mice with either construct and administering tamoxifen at the desired time to

recombine the LoxP-flanked 4D cassette together with the nuclear localization signal (nls) of GFP (or only the nls upon injection with control, GFP viruses). Note that this allowed to birthdate and discriminate between nuclear vs. cytoplasmic-GFP+ (GFP^{cyt}) neurons corresponding to infected, mature granule cells and newborn neurons generated from infected NSC after tamoxifen.

Animal surgery

Mice were kept in standard cages with a 12 h light cycle and provided with water and food ad libitum. Experiments were performed in 2-4 or 16 months old animals. For chronic manipulation of neurogenesis, the same cohorts of mice were serially tested throughout life. In all cases female *nestin*::CreRTt2 (Imayoshi et al., 2006) mice in the C57BL/6J genetic background were used. Isofluorane-anaesthetized mice were stereotaxically injected with 1 µl per hemisphere of viral suspension in the dentate gyrus as described in Artegiani et al. (2011, 2012) using a nanoliter-2000 injector (World Precision Instruments) and a stereotaxic frame Model 900 (Kopf Instruments) at ±1.6 mm mediolateral, –1.9 anteriorposterior, and –1.9 mm dorsoventral from bregma with a constant flow of 200 nl/min (**Fig. 2.2**). In acute experiments, recombination of the 4D cassette with the nuclear localization signal of GFP was achieved by oral administration of tamoxifen (Sigma) dissolved in corn oil (1:10) at 500 mg/kg body weight once a day for 4 days. When appropriate, animals were intraperitoneally injected with 100 µl of BrdU (Sigma) or EdU (Sigma) dissolved in PBS at 50 or 5 mg/kg concentration, respectively. Animals were either subjected to behavioural tests and/or anesthetized with pentobarbital and perfused transcardially with saline followed by 4% paraformaldehyde fixation in phosphate buffer (PFA). Animal procedures were performed in accordance to the local regulations (DD 24-9168.11-1/2011-11, TVV 13/2016 and HD 35-9185.81/G-61/15).

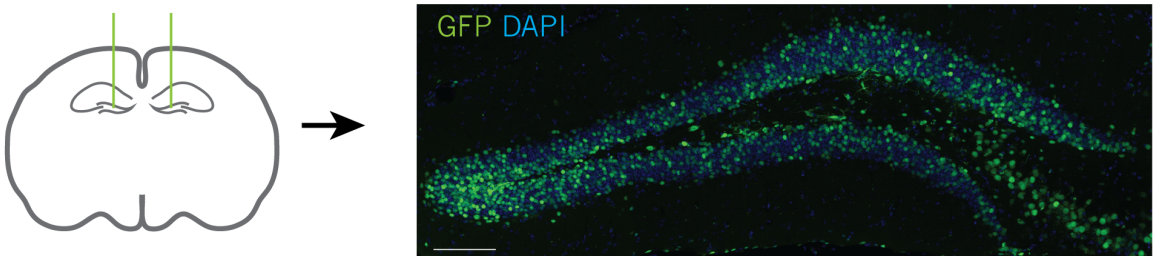

Fig. 2.2: Stereotaxic injection.

Injection of the lentiviral constructs by stereotaxic injection ensures the specific manipulation of hippocampal NSCs, as opposed to transgenic animals that could also be influencing the SVZ neurogenic niche.

Morris water maze

Mice were trained in the Morris water maze as described in Garthe et al. (2013) including 3 days of learning and 2 days of reversal in which the animals were trained to find the location of a hidden platform in a circular pool (1,89 cm diameter). Water was kept at 19-20°C and made opaque with a white non-toxic pigment. Before starting each experiment, mice were acclimated to the testing room for 30 minutes, dropped from different positions each day to favour spatial-hippocampal learning (Eichenbaum, 2000) and placed into a separate holding cage illuminated by a red warm light after every trial. Every day mice performed 6 trials with a minimum inter-trial time of 30 min. Each trial lasted a maximum of 2 min after which the mice were guided to the platform. The first day of reversal the position of the platform was changed to the opposite quadrant to assess re-learning. During the probe trials the platform was removed and the animals were dropped from a new starting point and kept in the water for 2 min. Swim paths were recorded and analysed using Ethovision (Noldus). Latency (s) and pathlength (cm) refer to the time and distance until the first encounter with the platform. The perseverance zone was defined as the circular area containing the former position of the platform, being the perseverance time defined as the time spent in that area during one trial. For the analysis of the swimming strategies

the swim paths were plotted and scored by two independent blind experimenters according to previous criteria (Garthe et al., 2009).

Barnes maze

The Barnes maze consisted of an elevated circular platform (1 m diameter) containing twenty holes (10 cm diameter) on the periphery of which one connected to an escape box. The maze was placed in a noise-isolated room with a strong light placed on top of the platform. After every trial, the platform and escape box were cleaned with 70% ethanol. All sessions were recorded and analysed using Ethovision (Noldus). Experiments consisted of three phases: habituation (1 day), learning (4 days) and reversal (3 days). Before starting each experiment, mice were acclimatized to the testing room for at least 30 minutes. Mice that had completed the test were then placed into a separate holding cage. On the habituation day (2 trials) mice were placed in the centre of the maze and given 4 min to freely explore the maze after which they were guided into the escape box and kept there for 30 s. During learning (3 trials of 3 min with 30 min minimum inter-trial interval) mice were trained to find a new position of the escape box. In the reversal phase (same number and duration of trials) the escape box was relocated 180 degrees opposite to the learning phase position. Mice were also subjected to probe tests (1 trial for 2 min, without escape box) at different times after the reversal phase (as indicated in the figures). Latency (s) was calculated as the time to the first encounter with the target position, whereas pathlength (cm) refers to the total distance travelled. For the analysis of the searching strategies, paths were plotted and scored independently by two experimenters blind of treatments and mouse grouping according to criteria adapted from previously reported Morris water maze tests (Garthe et al., 2009, 2013) (with the

exception that "thigmotaxis" was now considered as chaining; see examples in **Fig. 2.3**).

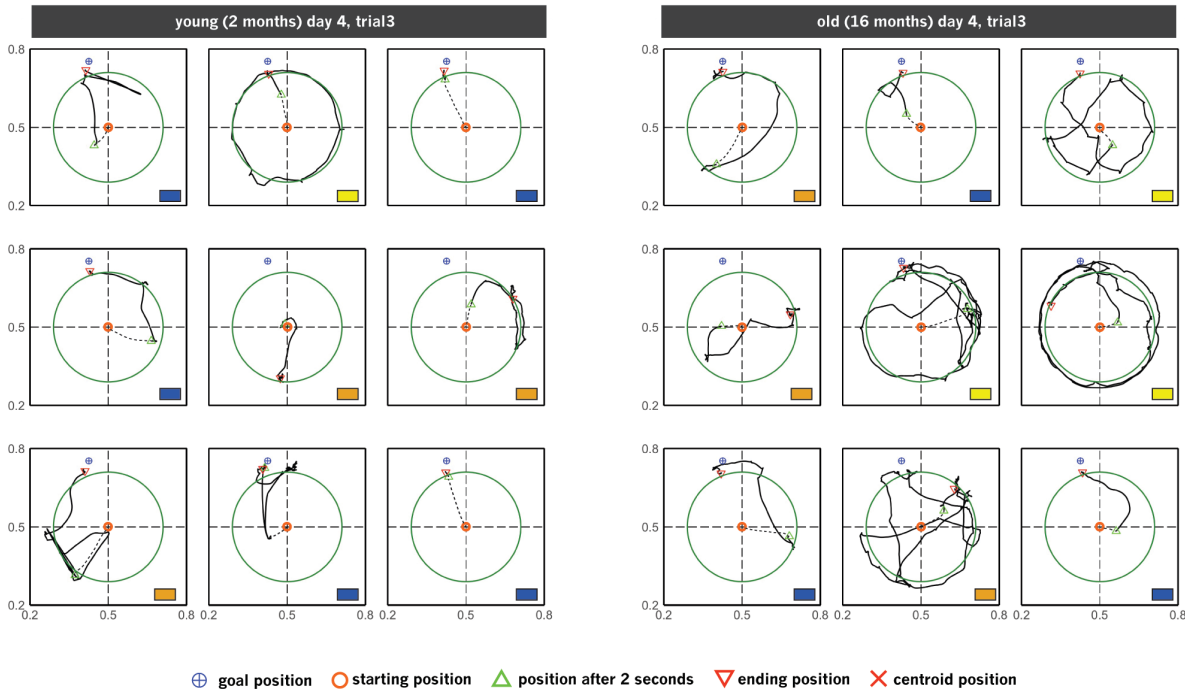

Fig. 2.3: Trajectories in the Barnes maze.

Examples of searching strategies used by mice and the corresponding colour code used to score them. Blue corresponds to allocentric/spatial or contextual strategies, whereas yellow represent egocentric/non-spatial or procedural navigation. Orange represents random strategies. Dashed lines represent the extrapolated path during the first 2 seconds of the trial that were not recorded by the software.

Contextual fear conditioning

Fear conditioning and contextual discrimination were assessed using a Multiconditioning System apparatus (TSE). Before each experiment, mice were acclimatized to the testing room for at least 30 min. Context A consisted on a soundproof shuttle box with two opposing transparent and two dark plexiglas walls, illuminated from above and ventilated. Context B consisted on a shuttle box with black-white striped plexiglas walls, without ventilation and only illuminated through doors left ajar. An electric footshock (2 s, 0.75 mA) was delivered through a steel grid 3 min after placing the mouse in the context A and

returned to a holding cage 15 s later. Mice placed in the context B mice for 3 min were never shocked. The grids and shuttle box were cleaned either with a 70% ethanol or non-alcoholic antiseptic solution prior to exposure to context A or B, respectively. A-B versus B-A exposures were performed as depicted in Fig. 1E with a minimum interval time between trials of 1 h. Levels of freezing were measured by the manufacturer's software allowing the assessment of discrimination ratio between contexts as follows: *(Freezing in A – B) / (Freezing in A + B)*, i.e. a score of 0 or 1 meant a complete lack or perfect discrimination, respectively. Probe trials were performed by exposing mice for 3 min to context A and B in different orders (as indicated in the respective figures). Re-training was performed in the same conditions as described for training.

Immunohistochemistry and cell quantifications

Brains were post-fixed overnight in 4% PFA at 4°C and cut in coronal 40-µm thick vibratome sections that were serially collected along the rostro-caudal axis of the hippocampus and stored at –20°C in cryoprotectant solution (25% ethylene glycol and 25% glycerol in PBS). Immunohistochemistry for different markers (**Table 2.7**) was performed stereologically 1 every 6 sections (resulting in a total of 10-12 analysed sections) after blocking and permeabilization with 10% donkey serum in 0,3% Triton X-100 in PBS for 1,5 h at room temperature. Primary and secondary antibodies were incubated in 3% donkey serum in 0,3% Triton X-100 in PBS overnight at 4°C. For BrdU detection sections were exposed to HCl 2M for 25 min at 37°C. EdU detection we performed following the manufacturer's guidelines (Click-iT EdU, Life technologies). DAPI was used to counterstain nuclei. Pictures were acquired using an automated Zeiss ApoTome or confocal (LSM 780) microscopes (Carl Zeiss) and maximal intensity projections of three optical sections (10 µm thick in total) were taken of the relevant brain area and

quantified using Photoshop CS5 (Adobe). Cellular quantifications were normalized per GFP+ cells (infected population) or area (total population, measured with Fiji 1.45b - ImageJ). Morphometric analyses of newborn neurons were performed by taking pictures through the entire thickness of the sections to include most processes that were later reconstructed using ImageJ.

Statistical analysis

For behavioural analyses, 8-13 mice were used per group (as indicated in the figures). Data were depicted as mean ± SEM (latency, pathlength, discrimination ratio), box-plots (contextual discrimination, perseverance and probe tests) or area charts (navigational strategies). Significance was calculated by i) 2-way ANOVA (evolution of performance over consecutive trials/days), ii) two-tailed paired or unpaired (as appropriate) Student's t-test (difference in performance in certain trials/days) or iii) Wald test of odd ratios assessed by logistic regression (navigational strategies). Cells quantifications and morphometric analyses were performed on at least 3 biological replicates per group with data reported as mean ± SD or box plots, respectively, with significance calculated by two-tailed unpaired Student's t test.

CHAPTER 3

RESULTS

Transient 4D overexpression was previously reported as a means to increase neurogenesis by selectively expanding the pool of hippocampal NSC, establishing a new and useful platform to specifically manipulate adult neurogenesis in vivo (Artegiani et al., 2011; **Fig. 2.1**). Briefly, during the first expansion phase 4D overexpression leads to an increase in NSC proliferation at the expense of neurogenic divisions and later on, in the differentiation phase upon 4D removal, the expanded pool of NSC gives rise to enhanced neurogenesis (**Fig. 3.1**).

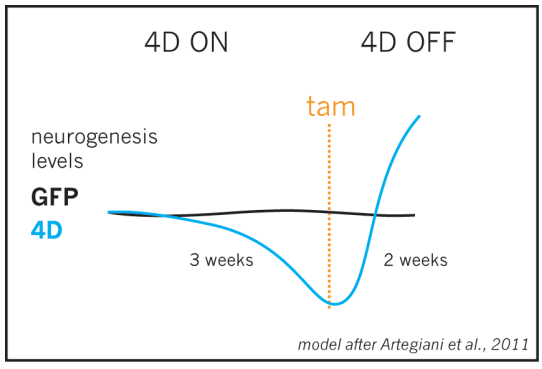

Fig. 3.1: 4D-increase in neurogenesis after NSC expansion reported by Artegiani et al. (2011).
Scheme depicting the effect of GFP (black) and transient 4D (blue) overexpression in neurogenesis levels, based on the results by Artegiani et al. (2011). Upon 4D overexpression, expansion of the hippocampal NSC at the expense of neurogenic divisions decreases neurogenesis levels, which are recovered and increased 2 weeks after tamoxifen administration in the differentiation, 4D OFF phase.

Importantly, some key questions important for the consideration of 4D as a valuable tool to study neurogenesis role and/or ameliorate age-related cognitive decline were so far unaddressed, including: i) survival and maturation of the 4D-generated neurons, ii) effect on cognition during youth, iii) effect of 4D manipulation in the aged hippocampus, iv) possible long-term effect of NSC expansion throughout life or v) potential effect on cognition during aging.

Thus, I first aimed to further investigate the consequences of acute NSC expansion and increased neurogenesis in young mice. To do this, I evaluated the neurogenic state at several differentiation times after tamoxifen by means of immunohistochemistry and morphological analysis of newly-generated neurons. Later on, I investigated the potential influence of enhanced neurogenesis in spatial learning behaviour.

3.1 4D-INCREASE IN NEUROGENESIS: TEMPORAL AND SPATIAL HOMEOSTHATIC REGULATION

Transient 4D overexpression results in a single wave of increased neurogenesis that matures at physiological rate

The effect of 4D overexpression during the expansion phase was comprehensively studied before (Artegiani et al., 2011). However, it was not known whether the increased NSC pool would rapidly exhaust during the differentiation, 4D OFF phase. Analysis of NSC proportion (Sox2+S100β- in SGZ) among the infected population (GFP+) revealed that the pool remained increased 2 weeks after tamoxifen in 4D brains relative to control infected with GFP viruses (8.51 ± 1.56 vs. 3.95 ± 1.67%, respectively; $p = 0.026$; **Fig. 3.2B**, left), suggesting that not all NSC differentiate after tamoxifen. The proportion of immature neurons (DCX+) in the GFP population was significantly increased by 2-fold in 4D brains relative to controls 2 weeks after tamoxifen, confirming previous results (6.76 ± 1.16 vs. 3.57 ± 0.96%, respectively; $p = 0.021$; **Fig. 3.1** and **Fig. 3.2B**, left; Artegiani et al., 2011). The special design of the viral construct, leading to the cleavage of the NLS after 4D recombination and cytoplasmic invasion of the GFP signal, allowed for the specific identification of the NSC progeny at different times after tamoxifen (**Fig. 2.1** and **Fig. 3.2C**). Consequently, the magnitude of the reported increase in neurogenesis was even more pronounced among the recombined population (GFP^{cyt}+: 3.36 ± 0.47 vs. 1.24 ± 0.70, $p = 0.012$; DCX+GFP^{cyt}+: 2.29 ± 0.48 vs. 0.62 ± 0.32% in 4D and GFP, respectively, $p = 0.007$; **Fig. 3.2B**, middle), indicating that 4D is highly specific in synchronizing new born neurons generation. This robust increase in neurogenesis was however not enough to observe significant differences in the total number of immature neurons in the DG

(infected and non-infected, DCX+/mm2: 1584.70 ± 605.26 vs. 1646.06 ± 503.66 in 4D and GFP respectively; $p = 0.90$; **Fig. 3.2B**, right).

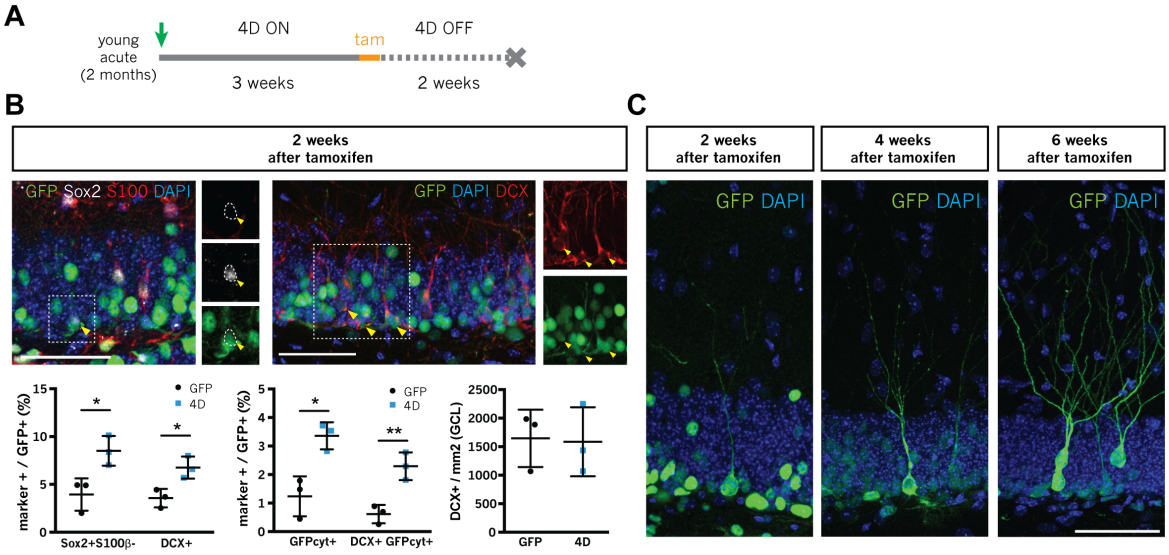

Fig. 3.2: **Increase in adult hippocampal neurogenesis after acute 4D overexpression in young mice (I).**
A, B. Experimental layout (**A**), immunohistochemistry fluorescence pictures (**B**, top) and quantifications (**B**, bottom) of cellular markers (as indicated) of newborn neurons in the hippocampus of 2 months old mice injected with GFP or 4D and analysed 2 weeks after tamoxifen administration. Insets (dashed boxes in B) are magnified. Cellular quantifications represent proportion of cells positive for markers within GFP+ infected cells or per area (mean ± sd; n = 3; B) in control (black) and 4D (blue). **C**. At different times after tamoxifen, cytoplasmic invasion of the GFP signal allowed for the identification and birthdating of the neural progeny derived from infected and recombined NSC. Scale bars = 100 µm (B, C). * $p < 0.05$; ** $p < 0.01$.

To address survival of the 4D-expanded cohort of neurons beyond their activity-dependent selection period (Sierra et al., 2010; Tashiro et al., 2006), cells were birthdated with BrdU during the week prior to tamoxifen and its co-localization with the neural marker NeuN in the DG was assessed 4 weeks after tamoxifen (**Fig. 3.3A**). The proportion of BrdU+NeuN+ among infected cells showed again a 2-fold increase relative to controls (0.64 ± 0.09 vs. 0.25 ± 0.05%, respectively; $p = 0.003$; **Fig. 3.3B** and **C**, left), suggesting that 4D-generated neurons survived at physiological rate. Accordingly, the proportion of GFP^{cyt} cells, generated at the moment of tamoxifen administration, was again doubled in 4D brains relative to controls (4.17 ± 0.71 vs. 1.95 ± 0.07%, respectively; $p = 0.005$; **Fig. 3.3B** and **C**,

right). When the proportion of immature neurons and NSCs were quantified 4 weeks after tamoxifen, no significant differences were observed between 4D and GFP infected brains (DCX+GFP+: 3.69 ± 0.67 vs. 4.15 ± 2.53%, respectively; $p = 0.89$; Sox2+S100β- in SGZ: 4.80 ± 1.90 vs. 4.27 ± 0.25%, respectively; $p = 0.66$; **Fig. 3.3D**), indicating that 4D-increase in neurogenesis is transient and suggesting the presence of homeostatic mechanisms restoring neurogenesis to basal levels 4 weeks after tamoxifen.

In order to morphologically characterize the expanded cohort of 4D-neurons, 4 weeks old GFP^{cyt} neurons were traced in GFP and 4D injected brains (**Fig. 3.3B**, right). As expected at this maturation point, both control and 4D-generated cells showed the first putative synaptic contacts in their dendritic arbours, and no difference was found in sholl profiles or dendritic lengths (**Fig. 3.3E**) suggesting that 4D-generated neurons mature and integrate physiologically. An additional morphometric analysis 6 weeks after tamoxifen, when newborn neurons reach a more mature integration pattern (Zhao et al., 2006), also revealed no difference in synaptic density relative to control (2.2 ± 0.34 vs. 2.3 ± 0.26 spines/μm in 4D and control, respectively; $p = 0.91$; **Fig. 3.3F**). At 12 weeks of age, 4D-generated neurons displayed mature granule cell morphology and connectivity pattern, with abundant dendritic arbourizations and visible synaptic connections in the hilar area and CA3 molecular layer (**Fig. 3.3G**).

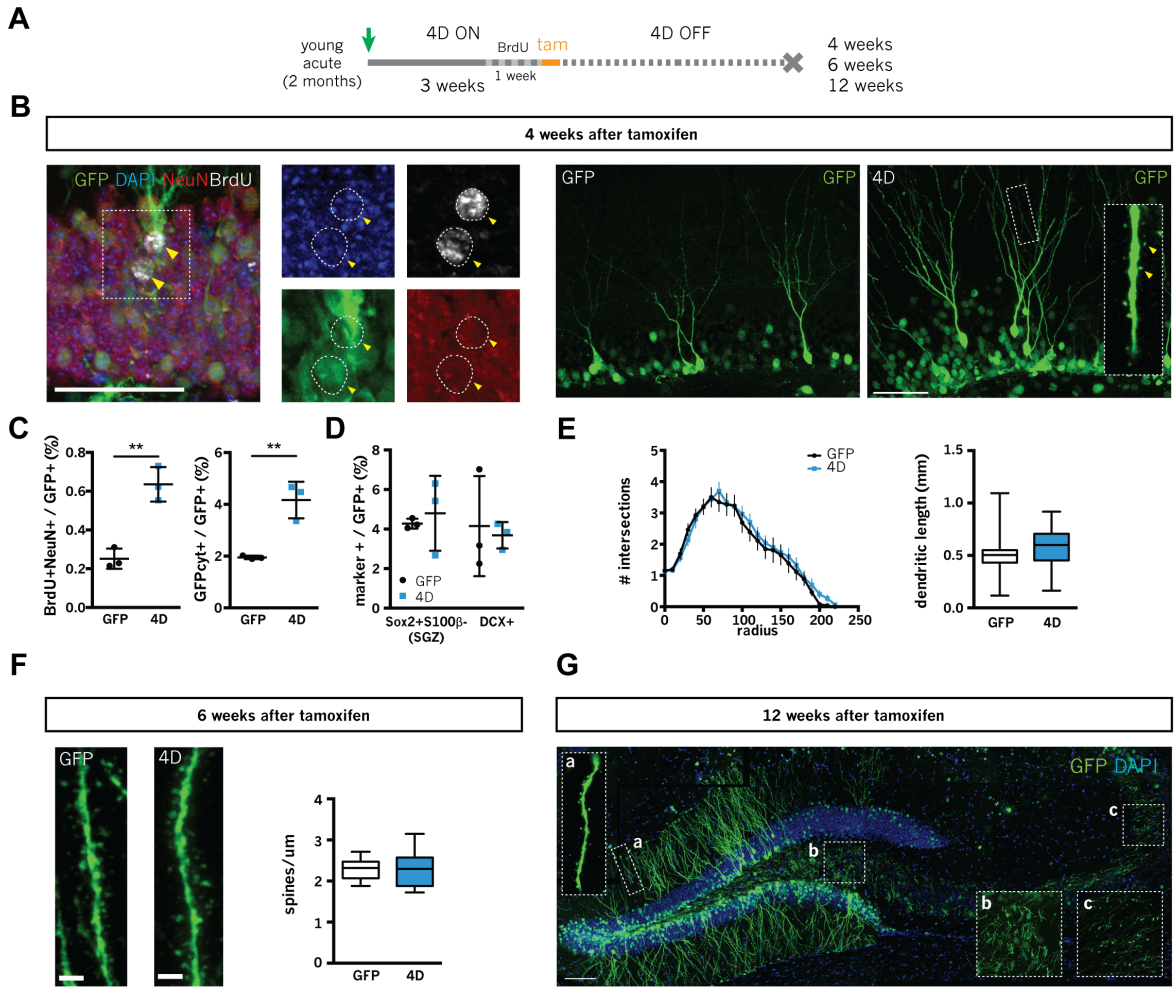

Fig. 3.3: Increase in adult hippocampal neurogenesis after acute 4D overexpression in young mice (II).

A. Experimental layout: after the expansion phase (4D ON, 3 weeks), mice were administered with tamoxifen (4D OFF) and neurogenesis was assessed 4, 6 and 12 weeks later. **B - E**. Fluorescence pictures (**B**), quantifications of cellular markers (as indicated; **C, D**) and morphometric analysis (**E**) of newborn neurons analysed 4 weeks after tamoxifen administration. **F**. Fluorescence pictures (left) and synaptic density of newborn neurons dendrites analysed 6 weeks after tamoxifen administration. **G**. DG 12 weeks after tamoxifen administration, depicting the main afferent and efferent structures of newborn neurons in the hippocampal circuit. Data represent proportion of cells positive for markers within GFP+ (mean ± sd; n = 3; C, D) and morphometric analysis represent sholl profiles (mean ± sem; E, left), dendritic lengths and synaptic densities (boxplots of at least 3 traced neurons per biological replicate; E, right and F) in control (black) and 4D (blue). Insets (dashed boxes in B and G) are magnified. Scale bars = 100 μm (B) and 2 μm (F). ** $p < 0.01$.

Altogether, these results show that 4D-increase in neurogenesis is transient, generating after tamoxifen administration a single increased wave of neurogenesis whose neurons physiologically mature, survive and integrate in the adult hippocampal system (**Fig. 3.4**).

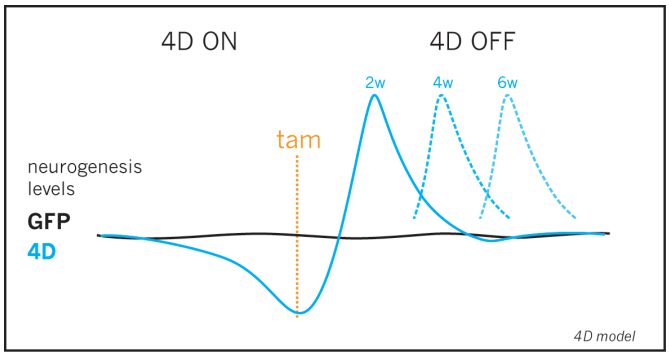

Fig. 3.4: Temporal regulation of 4D-increase in neurogenesis.

Scheme depicting the effect of GFP (black) and acute 4D (blue) overexpression in neurogenesis levels combining data from Artegiani et al. (2011) and data from the present thesis. Tamoxifen-dependent recombination of the infected NSC pool synchronizes differentiation leading to an expanded cohort of age-matched immature neurons 2 weeks later. During the following 2 weeks, this expanded wave integrates and matures at physiological rate, while neurogenesis returns to basal levels. As a consequence, acute 4D overexpression leads to the generation of a single wave of increased neurogenesis that is incorporated into the DG circuit.

Spatial homeostatic regulation of neurogenesis

Previous experiments by Artegiani et al. (2011), more focused on the expansion 4D ON phase, demonstrated that 4D-increase in neurogenesis is cell-intrinsic and NSC dependent (Artegiani et al., 2011). Data collected in this thesis during the differentiation, 4D OFF phase suggested the presence of additional cell extrinsic effects at later stages.

Four weeks after tamoxifen (**Fig. 3.5A**), when neurogenesis levels are normalized again in 4D brains, DCX+ cells showed a heterogeneous distribution mainly

concentrated in the less infected part of the GCL that highly contrasted with the homogeneous spread of these cells in GFP-infected animals (**Fig. 3.5B**). In line with this observation, the DCX and GFP signal along the GCL inversely correlated in 4D infected brains while they showed no correlation in the control group (average r = -0.481 and 0.002 respectively; $p < 0.001$; **Fig. 3.5C**). To evaluate the distribution of DCX+ cells along the GCL, the infected and non-infected compartment were defined based on the presence of GFP+ cells in the SGZ. Quantifications showed that the observed effect was due to an increase in the density of DCX+ cells in the non-infected GCL area of 4D animals compared to controls and the infected compartment in the same brains (ANOVA, $p = 0.007$; **Fig. 3.5D**). This pointed to an active neurogenic response in the non-infected area that was not observed at earlier time points (DCX+ ratio 4 weeks after tamoxifen: 0.39 ± 0.08 vs. 0.97 ± 0.27, $p = 0.024$; 2 weeks after tamoxifen: 1.06 ± 0.42 vs. 0.94 ± 0.22, $p = 0.68$, in 4D and GFP respectively; **Fig. 3.5E**). This cell-extrinsic effect was present through the whole extension of the DG, being more relevant in the ventral/temporal area since the infectivity is slightly smaller in this region (83% vs. 70% of dorsal vs. ventral DG infectivity; **Fig. 3.5F** and **G**).

These results suggest that phenomena occurring during the differentiation phase (when 4D is off) can actively influence the non-infected area producing a secondary local neurogenic reaction. This is important for future experiments, because it opens the possibility to use 4D also as a model to study the homeostatic mechanisms regulating neurogenesis in time and space. Additionally, the presence of such secondary neurogenic response could be used as a model to study anxiety-related behavior, which strongly relies in the ventral hippocampal function. (Bannerman et al., 2014; **Fig. 3.5H**).

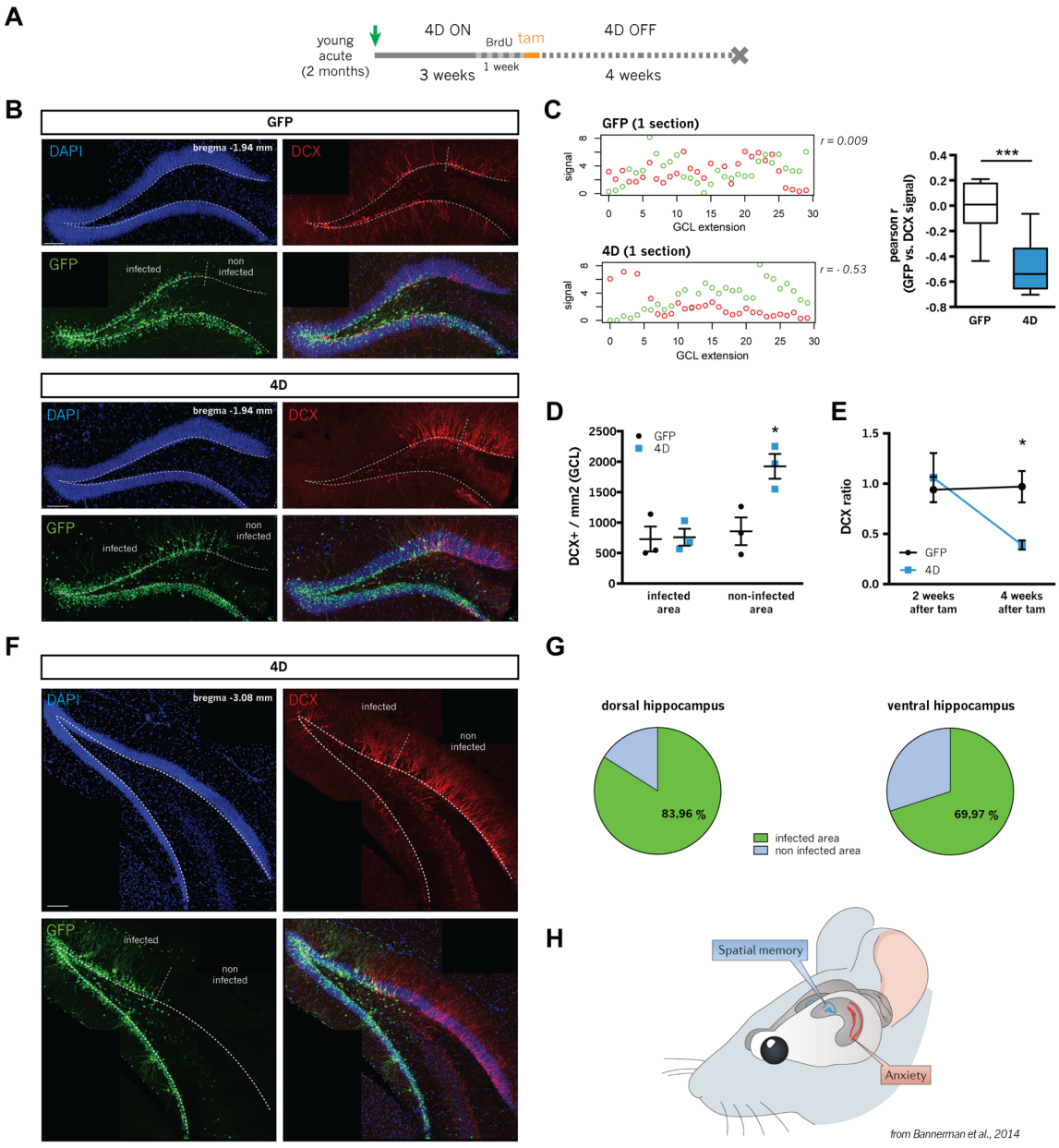

Fig. 3.5: Spatial homeostatic regulation of neurogenesis in the 4D system.

A - F. Experimental layout (**A**), fluorescence pictures (**B** and **F**), optic density of fluorescence signals (green for GFP and red for DCX; **C**) and cellular quantifications (**D** and **E**) in the whole extension or infected vs non-infected GCL compartments (as indicated) of the distribution of DCX+ cells assessed 4 weeks after tamoxifen administration. Data represents optic density of fluorescence per length and average pearson coefficient of at least 3 analysed sections per biological replicate (C, left and right respectively), total number of DCX+ cells per area (mean ± sd; n = 3; D) and the ratio of DCX+ cells in the infected and non-infected area 2 and 4 weeks after tamoxifen in GFP and 4D mice (as indicated; E). **G**. Percentage of infected and non-infected area in the dorsal and ventral hippocampus. Extension of the infected and non-infected GCL zone was made based on the presence or absence of GFP+ cells in the SGZ respectively. **H.** Cartoon depicting the functional and anatomical separation between the dorsal and ventral hippocampus, extracted from Bannerman et al. (2014). Scale bars = 100 µm. * $p < 0.05$; *** $p < 0.001$.

3.2 IMPROVING COGNITION DURING YOUTH: 4D-INCREASE IN NEUROGENESIS AND SPATIAL LEARNING

4D-increase in neurogenesis improves flexible learning

The transient nature of 4D-increase in neurogenesis provides an exceptional tool to study the influence of an expanded cohort of age-matched neurons in spatial learning and memory processes at different maturational times. In order to know whether 4D-increase in neurogenesis had an effect on spatial behaviour, mice were tested in a modified version of the Morris water maze. Briefly, animals were trained during 3 days, 6 trials per day, to find the hidden position of a platform in a water maze using visual cues. After this first learning phase, the position of the platform was changed (reversal) and re-learning was evaluated during the next 2 days. Importantly, reversal learning has been proposed to be a neurogenic-dependent indicator of flexible learning in mice (Garthe et al., 2009), although specific gain of function models supporting this hypothesis are missing.

Mice were tested 4 weeks after tamoxifen (**Fig. 3.6A**) when the increased cohort of 4D-generated neurons reached integration and is known to exhibit enhanced synaptic plasticity (Espósito et al., 2005; Gu et al., 2012b; Schmidt-Hieber et al., 2004). Analysis of the swimming pathlengths showed efficient learning for both learning phases, with no clear differences between groups (2-way ANOVA, learning phase, time: $F_{(2,42)} = 101.20$, $p < 0.0001$, group: $F_{(1,21)} = 0.0004$, $p = 0.98$, interaction: $F_{(2,42)} = 1.75$, $p = 0.19$; reversal phase, time: $F_{(1,21)} = 52.64$, $p < 0.0001$, group: $F_{(1,21)} = 0.85$, $p = 0.37$, interaction: $F_{(1,21)} = 3.44$, $p = 0.07$; **Fig. 3.6B**). Analysis of the swimming strategies revealed a progression from random and egocentric (such as chaining at a constant radius of the maze) towards allocentric, spatial learning strategies for both groups as the test proceeded (**Fig. 3.6C**).

However, 4D animals showed a 58% reduction in the use of perseverance strategies relative to controls after reversal (Odds ratio, OR = 0.28, p < 0.001; **Fig. 3.6C**, bottom), consistent with less visits to the former position of the platform on day 5 (time in perseverance zone: 3.23 ± 0.43 vs. 6.48 ± 0.89s respectively, t test p = 0.003; perseverance zone crossings: 1.71 ± 0.22 vs. 2.96 ± 0.35 respectively, t test p = 0.006; **Fig. 3.6D**) and suggesting profound improvements in reversal learning.

Immediately after the last trial of the test, a cohort (n = 3 per group) of these mice was sacrificed to assess neuronal activation by Arc immunostaining, showing 4D animals with improved flexible learning higher levels of activation among the infected granule neurons than controls (Arc+GFP+: 0.71 ± 0.07 vs. 0.54 ± 0.04%, respectively; p = 0.029; **Fig. 3.6E**). The rest of the animals were probe-tested for memory 2 weeks after the end of the test. I hypothesized that, if learning of the second platform position interfered with the first memory (**re-learning**), mice should show either preference for the second position or no preference in the case of catastrophic memory interference. On the other hand, if learning of the second position was independent from the first memory (**flexible learning**), mice should show preference for the position they remember better. During the probe test GFP-injected animals showed no preference between quadrants, suggesting memory interference, while 4D animals showed spatial preference the first platform position (%time in first vs. second target quadrant: 26.26 ± 6.87 vs. 20.13 ± 5.51%; t test, p = 0.62 and 31.64 ± 3.38 vs. 12.18 ± 3.89%, t test, p = 0.031 for GFP and 4D respectively; **Fig. 3.6F**). Interestingly, the first position had accumulated a higher use of spatial-dependent/contextual strategies in both groups during the test (day 3 vs. day 5 intragroup comparison: 58 vs. 31%, OR = 0.33, p < 0.001 and 70 vs. 42%, OR = 0.31, p = 0.002, for GFP and 4D respectively; **Fig. 3.6C** and **F**, left), suggesting a more solid learning.

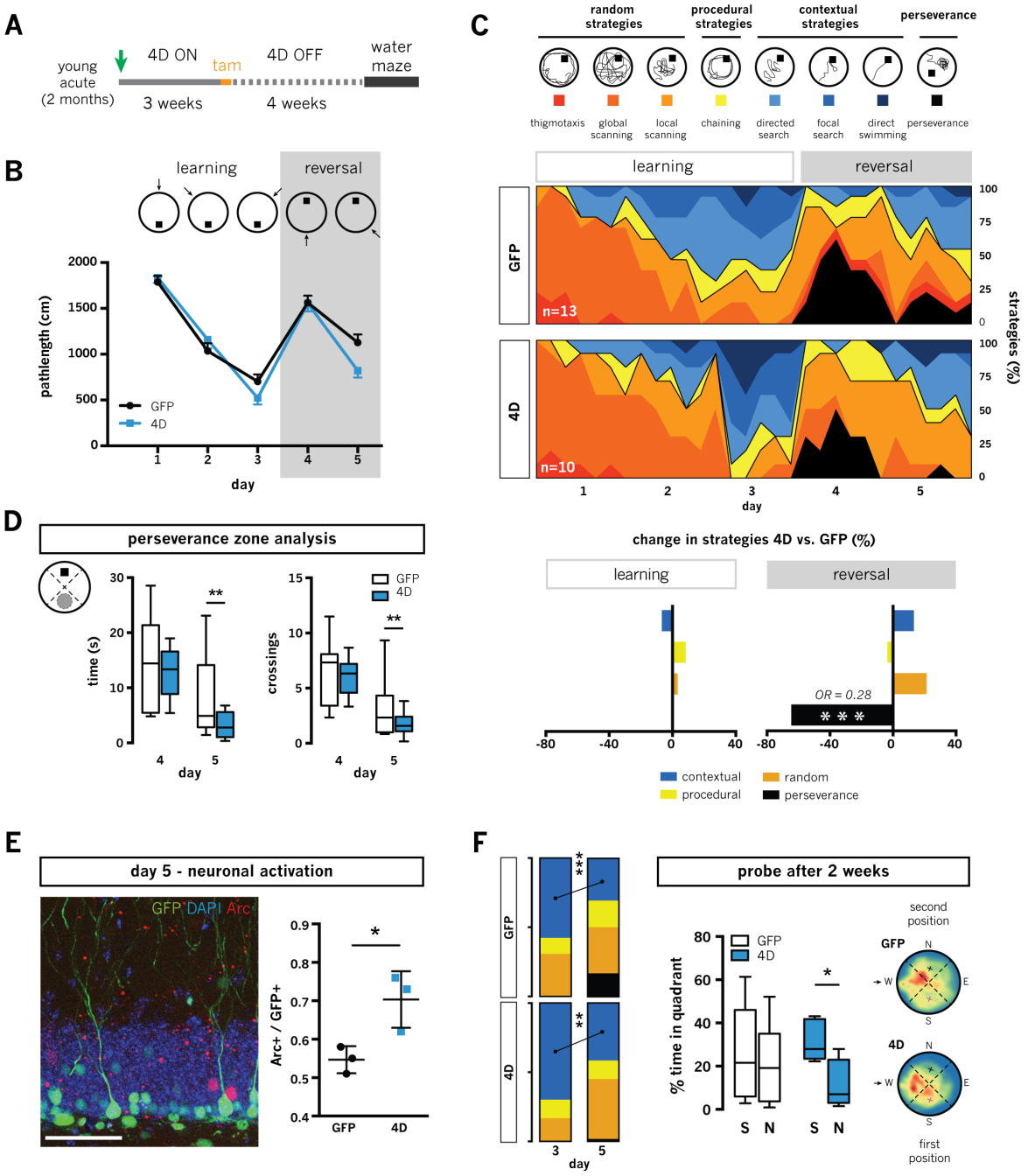

Fig. 3.6: 4D-increase in neurogenesis improves flexible learning.

A - D. Experimental layout (**A**), pathlengths (**B**), schematics, colour code, contribution and percentage change of learning strategies (**C**) and perseverance analysis after reversal (**D**) of GFP vs. 4D injected mice (as indicated, n=13 and 10 respectively) tested 4 weeks after tamoxifen administration in a modified version of the water maze (as in Garthe et al., 2009). **E**. Fluorescence picture and quantification of activated cells in the GCL after the last trial in day 5. **F**. Grouped strategies for days 3 and 5 in GFP and 4D animals (data from fig. 3.6C), showing an increased use of allocentric strategies during the first learning phase in both groups (left); on the right, probe memory test performance 2 weeks after the water maze with heat maps. Values represents mean ± sem (B) and mean ± sd (E). Boxplots represent time and crossings in perseverance zone (D) and percentage time per quadrant (F). Scale bar = 100 µm. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

These results indicate that an increased cohort of 4 weeks old neurons enhances flexible learning, correlating with a higher activation of the DG navigational circuit. Interestingly, mice with improved reversal learning showed preference for the first position of the platform in the probe test. This emphasises the conceptual difference between flexibility and re-learning, being 4D mice with increased neurogenesis able to flexibly learn something different without interfering with the pre-existing more solid memory.

Next, I assessed spatial learning in a different cohort of 4D and GFP-injected mice in the water maze 6 weeks after tamoxifen administration (**Fig. 3.7A**). These mice showed no difference in learning speed (2-way ANOVA, learning phase, time: $F_{(2,42)} = 31.89$, $p < 0.0001$, group: $F_{(1,21)} = 0.02$, $p = 0.88$, interaction: $F_{(2,42)} = 0.18$, $p = 0.83$; reversal phase, time: $F_{(1,21)} = 32.71$, $p < 0.0001$, group: $F_{(1,21)} = 1.67$, $p = 0.21$, interaction: $F_{(1,21)} = 0.25$, $p = 0.62$; **Fig. 3.7B**) or learning strategy use (**Fig. 3.7C**). This is consistent with the gradual loss of plasticity potential of newborn neurons after 4 weeks of age. This lack of effect also suggests that performance during spatial navigation is restored to control levels together with the normalization of the neurogenic state, with no observable effect of the ectopic neurogenic wave generated after 4D manipulation.

These results strongly indicate that maturational age is crucial for newborn neurons to influence the hippocampal system, and highlight the specific role of 4 weeks old neurons in flexible spatial learning. In the context of my work, these experiments establish a proof-of-principle showing that acute 4D manipulation increases neurogenesis and improve cognition in young mice. I next addressed the potential application of the 4D model in the context of aging and age-related cognitive decline.

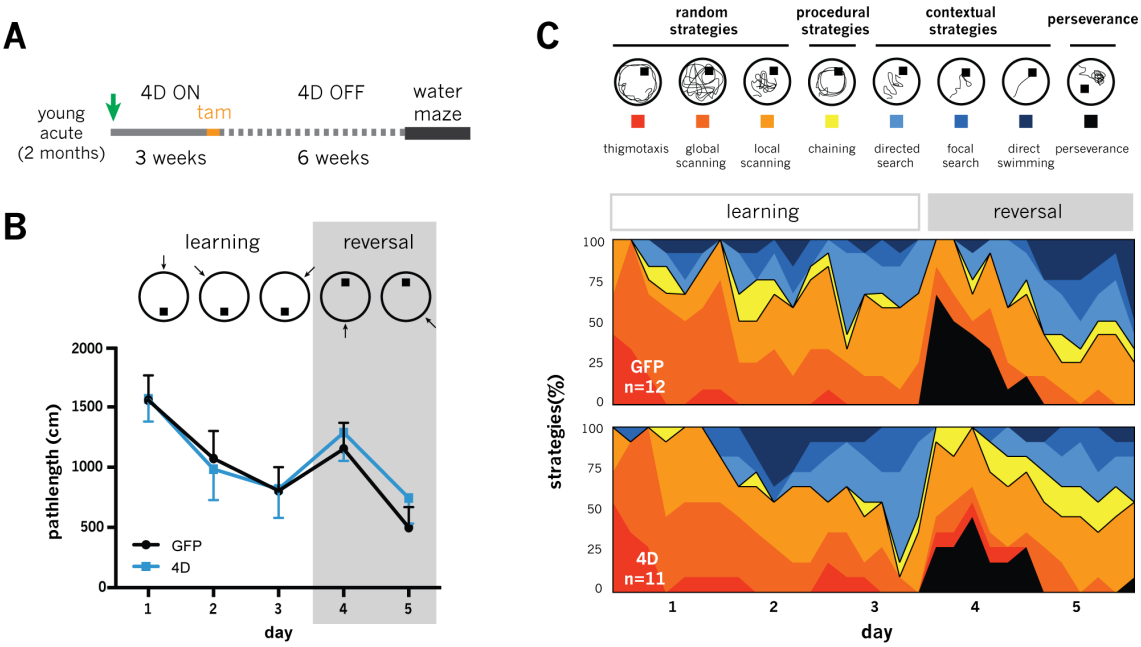

Fig. 3.7: 4D positive effect in learning is transient and depends on the maturation age of the newborn neurons. **A - C**. Experimental layout (**A**), pathlengths (**B**) and contribution of navigational strategies (**C**) of GFP and 4D injected mice (n = 12 and 11 respectively) and tested in the water mice 6 weeks after tamoxifen administration. Values represents mean ± sem (B).

3.3 AGE-RELATED DECAY IN HIPPOCAMPAL FUNCTION

Aging results in a switch from contextual to procedural learning

Evaluation of the learning strategies is essential because it underscores the cerebral process used by the brain to learn a given task (Squire, 2004). **Contextual or allocentric strategies** rely on the construction of a spatial map in the brain, are more dependent on the hippocampal function and create *place* memories, while **procedural or egocentric strategies**, consisting on the repetition of sequences, are more dependent on the striatal function and generate *response* memories or habits (Bach et al., 1999; Hartley et al., 2003; Iaria et al., 2003; Packard and McGaugh, 1996).

So far, the contribution of procedural strategies has been negligible since they are normally adopted during spatial learning in over-training conditions or after multiple reversals, as a way to save cognitive resources (Garthe and Kempermann, 2013). However, their use becomes more relevant during aging and age-related cognitive decline, where a progressive switch from allocentric to egocentric navigation learning has been shown to occur in a number of species from mice to humans (Leal and Yassa, 2015; Lester et al., 2017; Samson and Barnes, 2013). In order to model the age-related switch in learning strategies, 2 and 16 months old mice were tested in navigational and discrimination tasks and contextual versus procedural learning was assessed (note the adoption of the terms *contextual* and *procedural* instead of *allocentric* and *egocentric*, normally referred to navigation, in order to include contextual discrimination paradigms in the definition).

As testing old mice in the water maze is often confounded by a reduced physical endurance, a dry-land Barnes maze paradigm consisting on 4 days of learning and

3 days of reversal (3 trials per day) was used and learning strategies were evaluated. Young and old mice showed similar latency times during the two phases indicating that both groups learnt the task efficiently (**Fig. 3.8A**). However, old mice showed an approximately 50% increase in pathlengths during the whole test (ANOVA, time: $F_{(6,96)} = 9.50$, $p < 0.0001$, group: $F_{(1,16)} = 12.77$, $p = 0.002$, interaction: $F_{(6,96)} = 1.79$, $p = 0.11$; **Fig. 3.8A**, bottom), suggesting profound differences in the strategies used by the two groups. Both groups showed spatially-directed, contextual navigational strategies and procedural learning through chaining (**Fig. 3.8B**). However, young mice displayed a 2-fold increased contextual learning and decreased procedural strategies throughout both learning and reversal (OR, contextual = 1.85 and 2.28, chaining = 0.34 and 0.48, for learning and reversal; $p = 0.032$ and 0.002; 0.02 and 0.03, respectively; **Fig. 3.8B**, bottom). When animals were probe-tested for memory 4 weeks later, only young animals displayed a significant preference for the second position of the escape box (% time in first vs. second position quadrants: 15.61 ± 2.56 vs. $30.79 \pm 5.35\%$; $p = 0.003$) consistent with the acquisition of a *place* memory during reversal, whereas old mice showed no preference (% time in first vs. second position quadrants: 20.00 ± 4.51 vs. $32.99 \pm 4.45\%$; $p = 0.14$) and spent equivalent amount of time in all quadrants revealing the adoption of a *response* memory (**Fig. 3.8C**). Noteworthy, these changes in learning strategies during aging revealed that traditional parameters, such as latency (**Fig. 3.8A**; top), were alone insufficient to distinguish similarly efficient but cognitively different learning processes ultimately leading to the formation of memories of different nature.

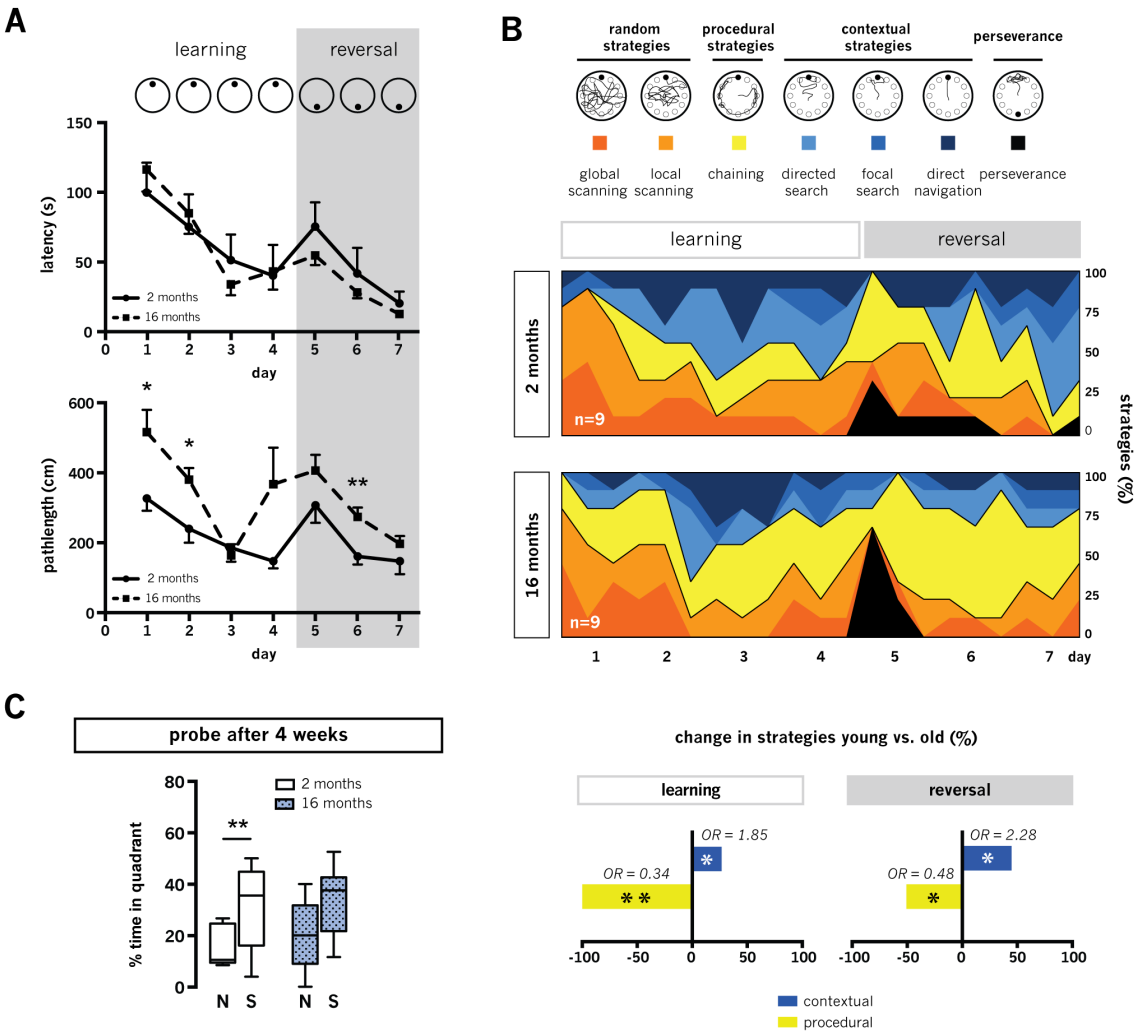

Fig. 3.8: Age-related change from contextual to procedural navigational strategies.

A - C. Latencies and pathlengths (**A**), schematics, colour code, contribution and percentage change of learning strategies (**B**) and performance in a memory probe test (**C**) of 2 vs. 16 months old un-manipulated mice (n = 9) performing a learning (4 days) and reversal (3 days) Barnes maze test (3 trials per day). Navigational strategies were scored and colour coded adapting criteria from the water maze (see **Fig. 2.3**). Values represents mean ± sem (A) and boxplots represent percentage time per quadrant (C). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

To corroborate these findings, a different cohort of young and old mice was used in a fear-conditioning test to investigate behavioural pattern separation and contextual memory, which in rodents are dependent on adult hippocampal neurogenesis (Sahay et al., 2011a). Training was performed for 3 days by exposure to a context A associated to a mild footshock and a similar context B without it (**Fig. 3.9A**). Although aging has also been associated to impairments in contextual

learning and memory interference (Leal and Yassa, 2015; McAvoy and Sahay, 2017), the identification of procedural learning strategies in discrimination tasks has not been reported. In order to evaluate to which extent mice distinguished context A irrespective of the presentation order (contextual learning) as opposed to freezing in the first context presented irrespective of contextual cues (procedural learning), the order of the two contexts was alternated each subsequent day during the discrimination phase (**Fig. 3.9A**).

After one day of training, both young and old mice showed comparable levels of conditioning and a similar degree of generalization between both contexts (**Fig. 3.9B**; day 1). Differences appeared during the discrimination phase (day 3-8) with young animals showing increasing levels of learning over time while old mice showed a poor discrimination for the entire test (ANOVA, time: $F_{(7,105)} = 1.23$, $p = 0.28$, group: $F_{(1,15)} = 35.18$, $p < 0.0001$, interaction: $F_{(5,75)} = 2.86$, $p = 0.020$; **Fig. 3.9C**). Interestingly, old mice displayed positive discrimination when context A was presented as the first context and no discrimination when it was presented as second context. As a result, the discrimination ratio of young and old mice was similar in days with an A-B order (**Fig. 3.9D**; left) while young mice discriminated better in days with a B-A order (day 6 and 8: $p = 0.008$; **Fig. 3.9D**; right). In fact, the average discrimination ratio of A-B vs. B-A days was equivalent in young mice but superior in A-B days in old mice (0.25 ± 0.08 vs. 0.39 ± 0.08 and 0.13 ± 0.03 vs. -0.03 ± 0.03 respectively; $p = 0.51$ and 0.018 respectively; **Fig. 3.9E**). In essence, age-related impairments seemed to be primarily due to a potential conflict between context and presentation order. In A-B days, both inputs were consistent and equivalent making the contextual learning of young as efficient as procedural learning of old mice. In contrast, in B-A days only a contextual learning strategy became effective revealing the impairment of old mice.

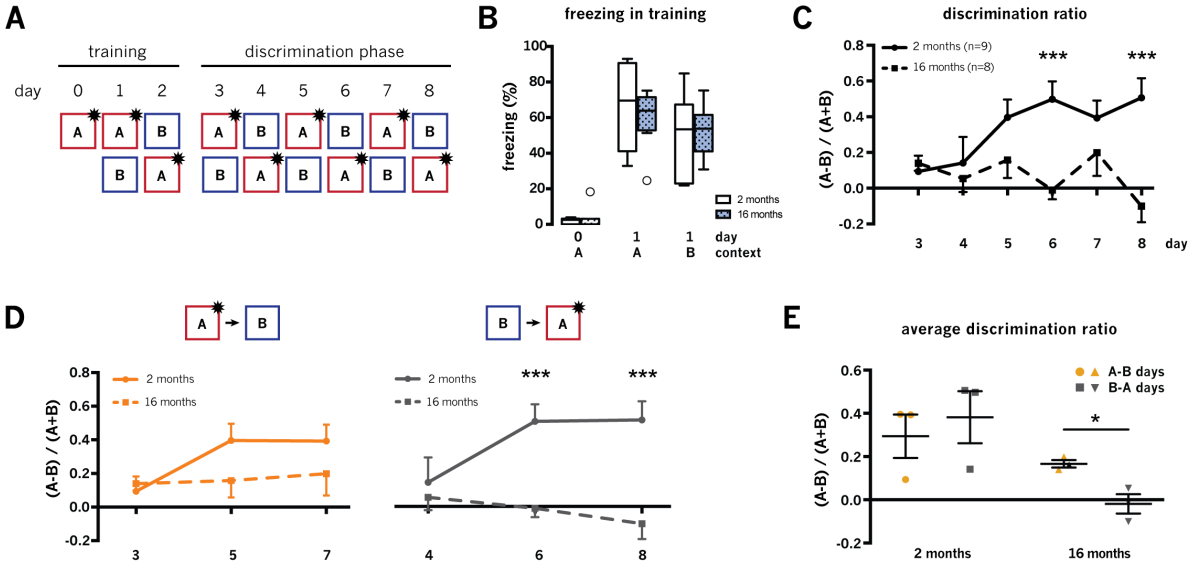

Fig. 3.9: Age-related switch in learning strategies in a contextual discrimination task.

A - E. Scheme of contextual discrimination protocol (**A**), and freezing during the training (**B**) and discrimination phase (**C**), in days with A-B or B-A presentation order (**D** and **E**) of 2 vs. 16 months old unmanipulated mice (n = 9 and 8 respectively) upon training (0-3 days) and testing (3-8 days) on a fear conditioning paradigm with context A (red) being associated with a footshock and alternating presentation order between contexts. Discrimination ratio is calculated as: *(freezing in A - freezing in B) / (freezing in A + freezing in B).* Boxplots represent freezing per context and day as indicated (B). Values represent means of each day (C and D) or group of days (E). * $p < 0.05$; *** $p < 0.001$.

Altogether, both navigation and contextual discrimination of young versus old mice indicated that aging is associated with a switch from contextual to procedural learning.

Aging modifies the neural activation pattern associated to contextual memories

Malfunction of the declarative memory system, especially the hippocampus, is often proposed as one of the major causes leading to the age-related decay in contextual memory systems and switch towards procedural learning (Hedden and Gabrieli, 2004; Leal and Yassa, 2015; Lester et al., 2017; Samson and Barnes, 2013). Although impairments at the hippocampal modulatory and electrophysiological level have been reported, more evidence explaining the switch in learning strategies during aging are needed. Here, I assessed if impairments at the circuit level described in the literature could be leading to weakened brain representations (engrams) of contextual memories in old mice. To address this question, an additional cohort of 2 and 16 months old mice was subjected to fear conditioning sacrificed right after re-exposure to the same context the following day to assess neuronal activation in different brain areas by c-Fos immunostaining.

Consistent with the previously observed phenomena, no difference in freezing behaviour was observed between young and old mice after one day of conditioning, meaning that both groups were able to express the memory efficiently. However, strong differences in neuronal activation after re-exposure were found between young and old animals in brain areas important for the contextual conditioning system, such as the suprapyramidal blade of the DG (DG-SB), hippocampal CA1, basolateral amygdala (BLA) and central amygdala (CeA; DG-SB, 638.96 ± 19.07 vs. 607.44 ± 3.52; CA1, 1003.30 ± 64.73 vs. 872.25 ± 38.15; BLA, 381.07 ± 22.92 vs. 554.30 ± 19.53; CeA, 456.64 ± 28.67 vs. 532.67 in young vs. old, respectively; all, $p < 0.05$) together with a strong tendency in the dorsomedial striatum (STR DM; 614.68 ± 72.39 vs. 508.91 ± 61.48 in young vs. old, $p = 0.067$; **Fig. 3.10**), supporting previous findings (Barnes et al., 2000;

Cleland et al., 2017; Penner et al., 2011). These results suggest that, although the behavioural output is comparable, differences in memory circuits between young and old mice can be seen early after exposure to a simple contextual task and prior to the adoption of a stable learning strategy. This different activation pattern, especially in the hippocampal structures encoding contextual information, could be the substrate of an impaired memory representation interfering with hippocampal learning and leading to the use of less contextually-dependent strategies in old mice.

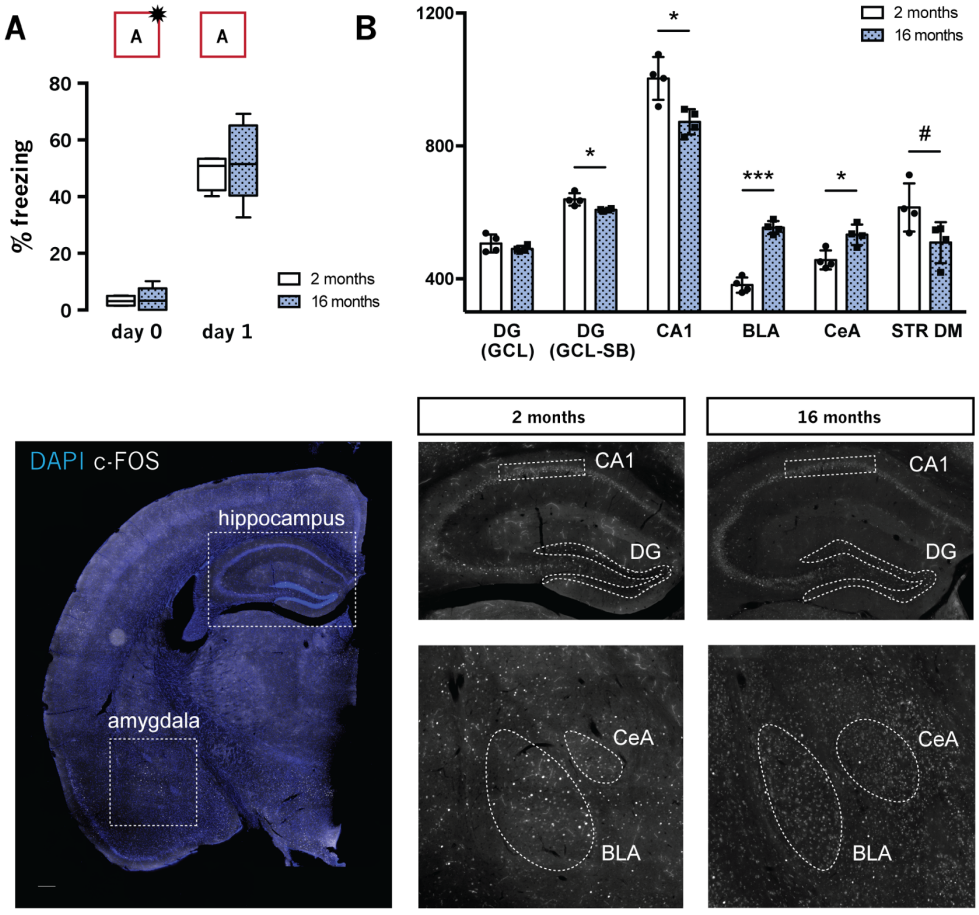

Fig. 3.10: Memory engram formation changes with age.

A - B. Freezing after fear conditioning (**A**) and cellular quantifications (**B**) and pictures of c-Fos+ cells after memory expression in different brain areas in un-manipulated young vs. old mice. Boxplots represent freezing per day and group as indicated. Values represent c-Fos+ cells per brain structure area in young and old mice, as indicated (mean ± sd; n = 4 and 5, respectively). Scale bar = 100 µm. DG-SB = suprapyramidal blade of the DG; STR DM = dorsomedial component of the striatum; BLA = basolateral amygdala; CeA = central nucleus of the amygdala; # $p < 0.1$; * $p < 0.05$; *** $p < 0.001$.

Therefore, I hypothesized that approaches rescuing the deficits in contextual processing and boosting hippocampal function could help to ameliorate the effects of aging in the brain. Although the role of adult hippocampal neurogenesis in contextual engram formation is not yet well understood (Anacker et al., 2015), neurogenesis has been proposed as a valuable target to rejuvenate and optimize memory circuits during aging (McAvoy and Sahay, 2017). Given the positive results obtained after 4D manipulation in young mice, I decided to investigate to potential benefits of 4D in rejuvenating hippocampal function and contextual-dependent learning during aging.

3.4 4D MANIPULATION DURING AGING: REJUVENATING THE BRAIN

4D NSC expansion compensates age-related decline in neurogenesis throughout life

Together with its strong effects on learning and memory, aging is also known to be one of the most important negative regulators of adult hippocampal neurogenesis (Aimone et al., 2014; Kempermann, 2011; Lee et al., 2012). Adult-generated neurons dramatically decline during adulthood, showing old mice only a small fraction of the newborn neurons levels typical of young mice (DCX+: 58.26 ± 21.04 vs. 1646.06 ± 503.66 respectively; $p = 0.005$; **Fig. 3.11**). This age-related decline in neurogenesis has been proposed to directly depend on either the depletion or less proliferative potential of the aging NSC pool (Bonaguidi et al., 2011; Encinas et al., 2011). In either case, the specific effect of 4D in NSC expansion and its positive impact on cognition described during youth paved the way for new experiments attempting to compensate the age-related decline in neurogenesis and ultimately rescue the switch from contextual to procedural learning.

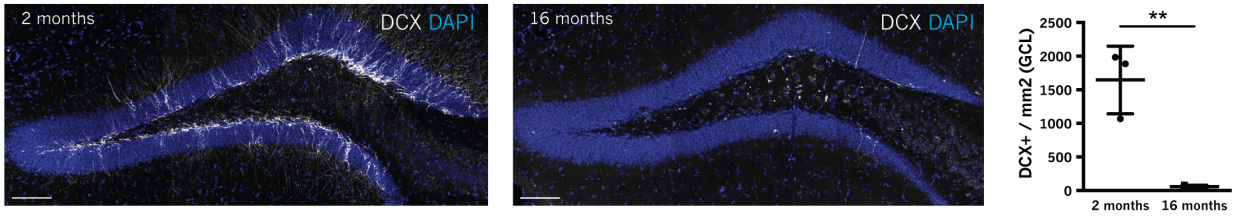

Fig. 3.11: Age-related decline in neurogenesis.

Fluorescence pictures (left) and quantifications of DCX+ immature neurons in the DG of young and old mice, depicting the age-related decline in adult hippocampal neurogenesis. Values represent positive cells per area in young and old mice, as indicated (mean ± sd; n = 3). Scale bars = 100 μm. ** $p < 0.01$.

First, I assessed whether 4D would still be effective in rescuing neurogenesis acutely in old mice or chronically throughout life. Acute 4D overexpression in 16 months old mice for 3 weeks (**Fig. 3.12A**, top) resulted in a 6-fold increase in overall proliferation and active NSC among infected (GFP+) cells relative to controls (EdU+: 0.77 ± 0.22 vs. 0.16 ± 0.11% and Sox2+/EdU+: 0.53 ± 0.14 vs. 0.08 ± 0.07%; $p = 0.013$ and $p = 0.008$, respectively) that was paralleled by a doubling in NSC in the SGZ (Sox2+/S100–: 6.17 ± 1.1 vs. 3.73 ± 0.74%; $p = 0.033$; **Fig. 3.12A**, bottom). To know whether NSC expansion could lead to increased neurogenesis in old mice, 4D overexpression was followed by either 2 or 4 weeks of differentiation upon tamoxifen administration (**Fig. 3.12B**, top). This led to a doubling in immature neurons 2 weeks after tamoxifen in 4D-infected brains relative to controls (DCX+: 0.35 ± 0.06 vs. 0.19 ± 0.05%, respectively; $p = 0.032$) that was again more evident in the recombined NSC progeny (DCX+GFP^{cyt}+: 0.09 ± 0.03 vs. 0.01 ± 0.01%, respectively; $p = 0.016$; **Fig. 3.12B**, left). Once more, neurogenesis in 4D-treated mice returned to basal levels 4 weeks after tamoxifen (DCX+: 0.66 ± 0.40 vs. 0.58 ± 0.49%; $p = 0.83$; **Fig. 3.12B**, right). In this case, no ectopic reaction could be observed in the non-infected DG compartment. The proportion of mature neurons EdU-birthdated 4 weeks prior to tamoxifen showed a 3-fold increase relative to controls (EdU+/NeuN+: 0.24 ± 0.04 vs. 0.08 ± 0.08%; $p = 0.037$; **Fig. 3.12B**, right) confirming the physiological survival of the expanded wave of 4D-generated neurons also in old mice. Morphometric analyses at 4 and 6 weeks post-tamoxifen showed that arborization, dendritic length and synaptic density of 4D-derived neurons were undistinguishable from controls (**Fig. 3.12C** and **D**). Hence, transient 4D overexpression had very similar consequences in young and old mice, doubling the levels of neurogenesis relative to control in both conditions and generating a single wave of expanded newborn neurons.

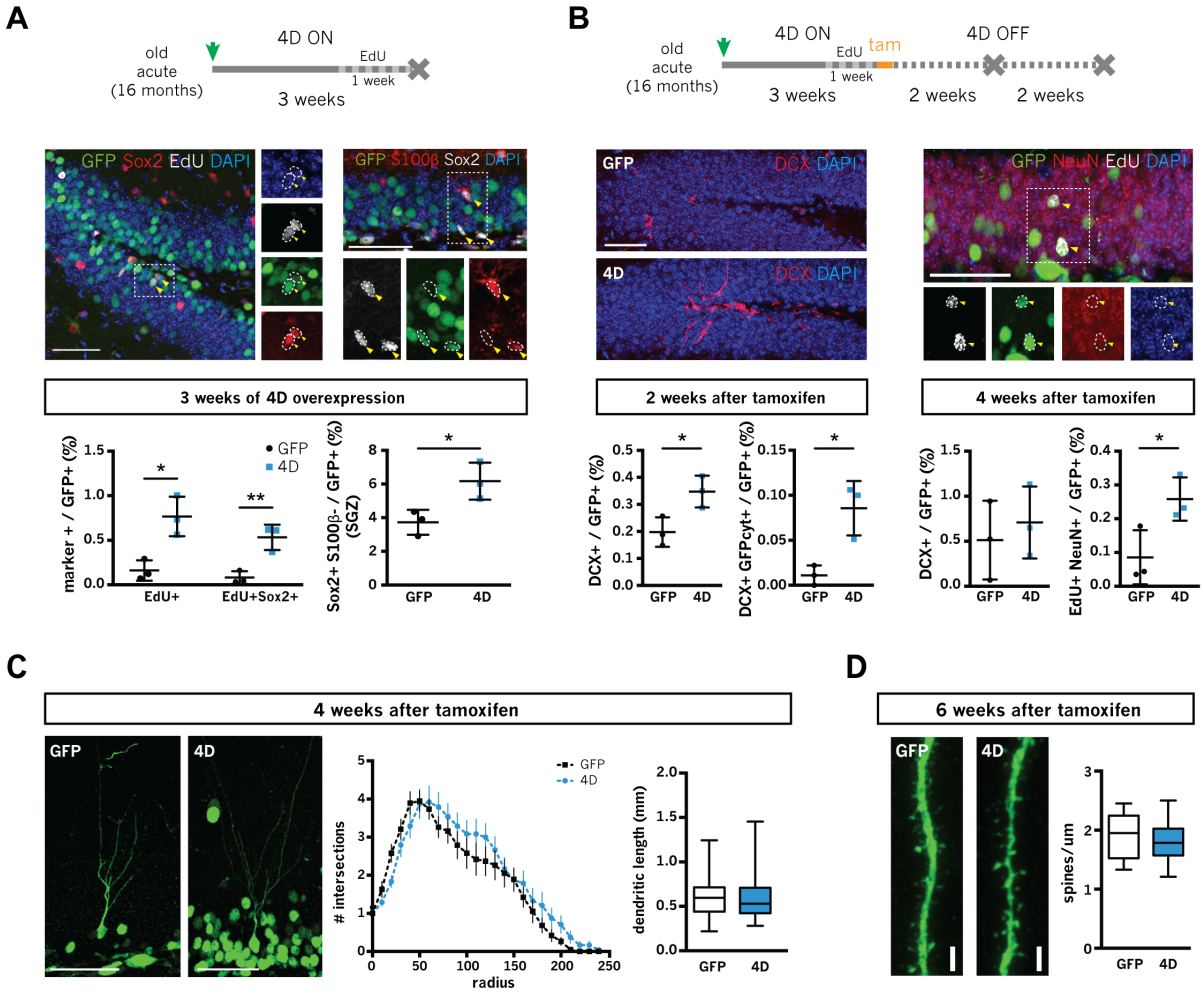

Fig. 3.12: Acute 4D manipulation increases neurogenesis in the old DG.

A, B. Experimental layout (top), fluorescence pictures (middle) and cellular quantifications (bottom) of markers in the DG of GFP and 4D injected 16 months old mice, as indicated, depicting the neurogenic state 3 weeks after 4D overexpression (4D ON; **A**) and 2 and 4 weeks after tamoxifen administration (4D OFF; **B**). **C, D**. Morphometric quantifications (**C**) and synaptic densities (**D**) of GFP and 4D-generated neurons 4 and 6 weeks after tamoxifen, respectively. Data represent proportion of cells positive for markers within GFP+ (mean ± sd; n = 3; A, B) and morphometric analysis represent sholl profiles (mean ± sem; C, left), dendritic lengths and synaptic densities (boxplots of at least 3 traced neurons per biological replicate; C and D) in control (black) and 4D (blue). Insets (dashed boxes in A and B) are magnified. Scale bars = 100 µm (A, B and C, left) and 2 µm (C, right). * $p < 0.05$.

Finally, in order to know whether it is possible to get a constant effect beyond the first increased wave of neurons, I sought to enhance neurogenesis throughout life by continuous 4D overexpression in 4 months-old mice for one year (i.e., without tamoxifen administration) (**Fig. 3.13A**, top). Three months after viral

infection, a doubling in neurogenesis in 4D relative to GFP-expressing control mice was observed (DCX+: 2.27 ± 0.20 vs. 1.18 ± 0.27, respectively; $p = 0.005$; **Fig. 3.13A**), comparable to the increase after a transient 4D manipulation in young (**Fig. 3.2B**) or old (**Fig. 3.12B**, left) mice. This 4D-induced increase in immature neurons was followed by a doubling in the proportion of mature neurons BrdU-birthdated 2 months prior to sacrifice (BrdU+/NeuN+: 1.19 ± 0.11 vs. 0.53 ± 0.15, respectively; $p = 0.003$; **Fig. 3.13B**) corroborating the physiological maturation and survival of 4D-derived neurons also after this chronic approach. Remarkably, quantifications in 10, 13 or 16 months old mice showed increased levels of neurogenesis throughout life (DCX+, ratio of 4D/GFP = 2.3, 3.7 and 4.2 respectively; all timepoints $p < 0.025$; **Fig. 3.13A** and **D**). Analysis of the Sox2 compartment revealed that the effect of 4D in NSC expansion was still visible 9 months after injection (Sox2+S100β- in the SGZ: 7.52 ± 0.71 vs. 4.96 ± 0.41% in 4D and GFP respectively, $p = 0.006$; **Fig. 3.13C**), together with a positive trend in the proportion of astrocytes (Sox2+S100β- in the GCL: 3.72 ± 0.9 vs. 2.19 ± 0.48% in 4D and GFP respectively, $p = 0.059$; **Fig. 3.13C**) that is consistent with the terminal NSC-astrocytic differentiation described before (Encinas et al., 2011). Importantly, no tumorigenic effect was observed in any of these experiments.

Altogether, these results show that 4D overexpression can compensate the natural decline in NSC activity and hippocampal neurogenesis acutely in old mice or chronically throughout life. These two approaches are different in that the acute and chronic 4D manipulations result in an increased cohort of age-matched neurons and an increased basal state of neurogenesis respectively. Each of them allow the study of the role of neurogenesis in cognition during aging from a different perspective.

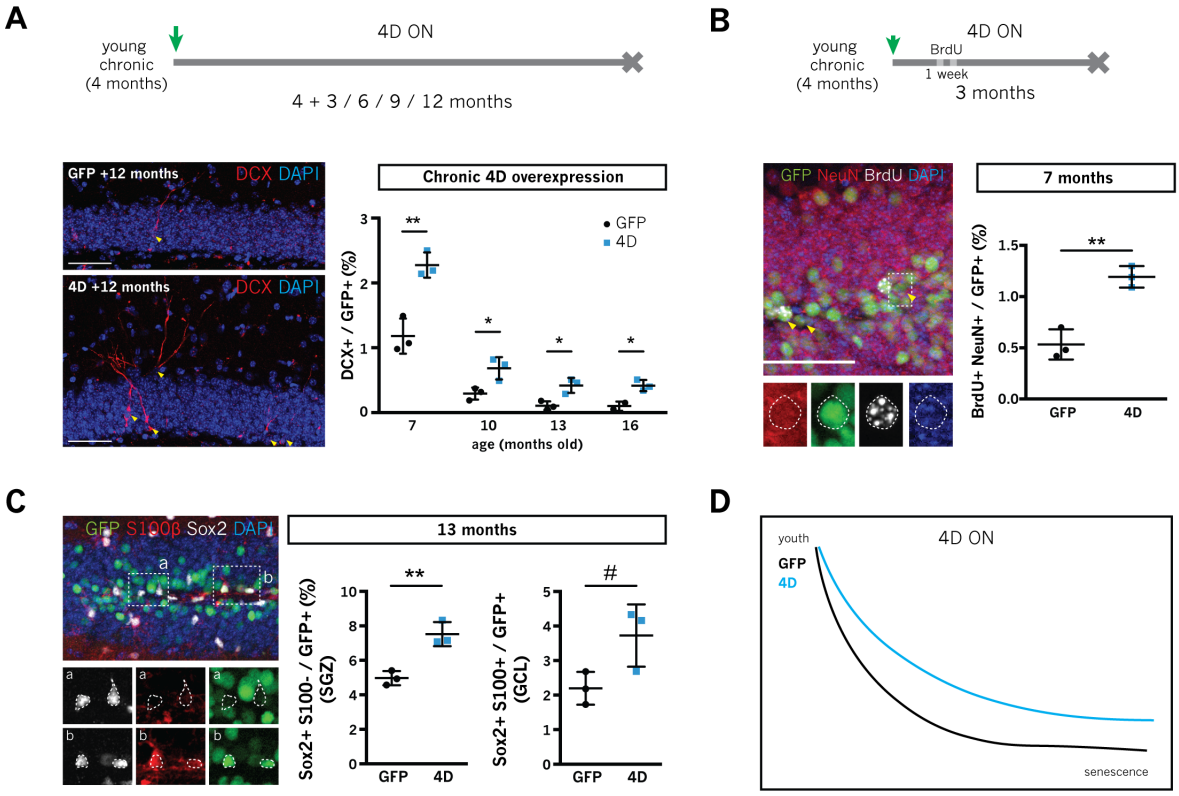

Fig. 3.13: Chronic 4D manipulation increases neurogenesis throughout life.

A - C. Experimental layout (top), fluorescence pictures (left) and cellular quantifications (right) of markers in the DG of GFP and 4D mice injected at 4 months of age and analysed at different times after the injection, as indicated, depicting the levels of neurogenesis throughout life (**A**), integration of newborn neurons (**B**) and long-term state of the Sox2+ compartment (**C**). Note that in this chronic manipulation no tamoxifen and therefore no 4D OFF phase is present. **D**. Scheme showing the age-related decrease in neurogenesis showed by GFP injected mice and its attenuation by sustained 4D overexpression throughout life. Data represent proportion of cells positive for markers within GFP+ (mean ± sd; n = 3). Insets (dashed boxes in B and C are magnified. Scale bars = 100 μm (A, B and C, left) and 2 μm (C, right). # $p < 0.1$; * $p < 0.05$; ** $p < 0.01$.

An acute increase in neurogenesis rejuvenates navigational learning strategies and spatial memory

To investigate possible cognitive effects of an acute increase in neurogenesis in aged mice, I induced NSC expansion in 16 months old mice for 3 weeks and subjected them to the Barnes maze 4 weeks after tamoxifen, when the increased wave of 4D-generated neurons reaches integration (**Fig. 3.14A**).

4D-treated mice showed no apparent improvement relative to GFP-injected controls in pathlengths and latency during the test (**Fig. 3.14B**), while showing a 2-fold increase in both parameters during the first day after reversal (day 5; both $p < 0.05$; **Fig. 3.14B**) due to an increased perseveration at the previous position of the escape box (time, $p = 0.051$; crossings, $p = 0.010$; **Fig. 3.14C**). Remarkably, analysis of the searching strategies revealed that this a priori negative effect was due to a doubling in the use of contextual (OR = 2.17; $p = 0.038$) at the expense of procedural (OR = 0.30; $p < 0.001$) strategies relative to controls during the first learning phase (**Fig. 3.14D**). This increased use of spatial learning explained why reversal affected only 4D-treated mice, since chaining in controls was always effective irrespective of the escape box position resulting in a constant pathlength and latency (**Fig. 3.14D**). In addition, 18 months old control mice showed decreased contextual learning also relative to 16 months old untreated mice (OR = 0.28; $p < 0.001$) showing that the loss in hippocampal function in these 2 months was as substantial as the one observed over more than one year from 2 to 16 months (compare **Fig. 3.8B** and **Fig. 3.14D**).

During reversal, 4D-treated mice continued to develop contextual, spatial strategies more efficiently than controls leading, in the last day, to a significant increase in contextual (OR = 3.25; $p = 0.036$) and reduced procedural (OR = 0.30; $p = 0.027$) strategies (**Fig. 3.14D**). A probe trial 2 weeks later showed that GFP-injected mice had no preference for neither the learning nor reversal position of the escape box (time in first vs. second quadrant: 24.8 ± 2.3 vs. 25.0 ± 3.0%; $p = 0.955$) while 4D-treated mice displayed a 2-fold increased preference for the latter (17.9 ± 1.8 vs. 33.5 ± 3.3%; $p = 0.011$; **Fig. 3.14E**) consistent with the acquisition of a response navigational memory in control and switch towards place memory upon increased neurogenesis. Although previous works also reported a positive effect of increased neurogenesis in memory during aging, this is the first

evidence relating this effect to the adoption of a more spatial and hippocampus-dependent contextual learning strategy.

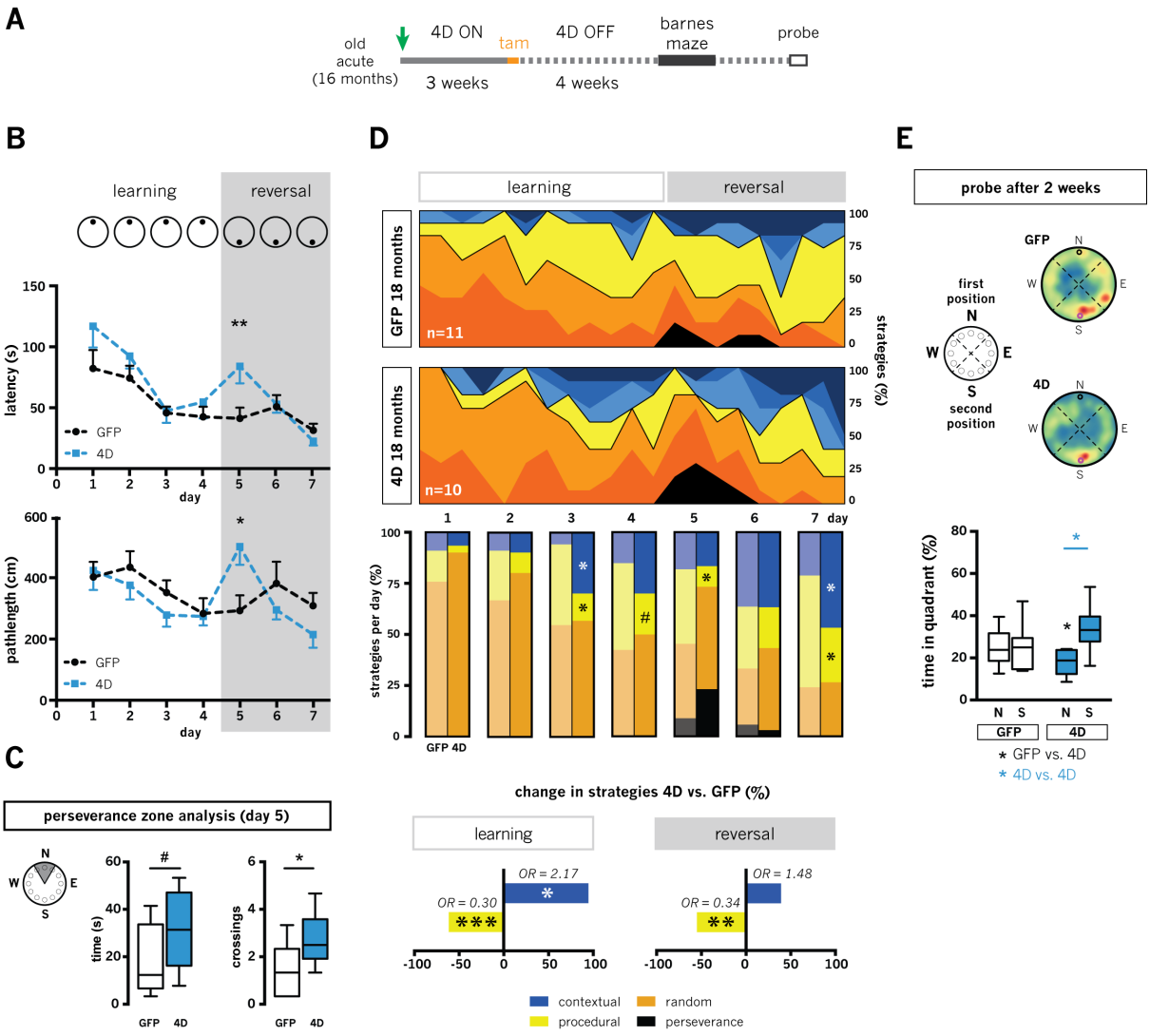

Fig. 3.14: An acute increase in neurogenesis rejuvenates learning strategies in old mice.

A - E. Experimental layout (**A**), latencies and pathlengths (**B**), perseverance behaviour (**C**), learning strategies analysis by group and day, together with percentage change (**D**) and memory performance in a probe test (**E**) of GFP vs. 4D injected old mice (16 months old; n = 11 and 10 respectively) tested 4 weeks after tamoxifen administration in the Barnes maze. Values represents mean ± sem (B). Boxplots represent time and crossings in perseverance zone (C) and percentage time per quadrant (E). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

A different cohort of GFP and 4D-injected mice was subjected to contextual discrimination to corroborate these findings. Performance of control mice

confirmed the previously observed effects of aging (**Fig. 3.9C**) with positive discrimination in A-B but not B-A days (**Fig. 3.15C** and **D**). Interestingly, this effect was more pronounced and significant in controls (0.19 ± 0.08 vs. 0.00 ± 0.07, respectively; $p = 0.013$) than in 4D-treated mice (0.10 ± 0.05 vs. –0.02 ± 0.07; $p = 0.10$; **Fig. 3.15C** and **D**) suggesting a reduced bias for presentation order and procedural learning upon increased neurogenesis.

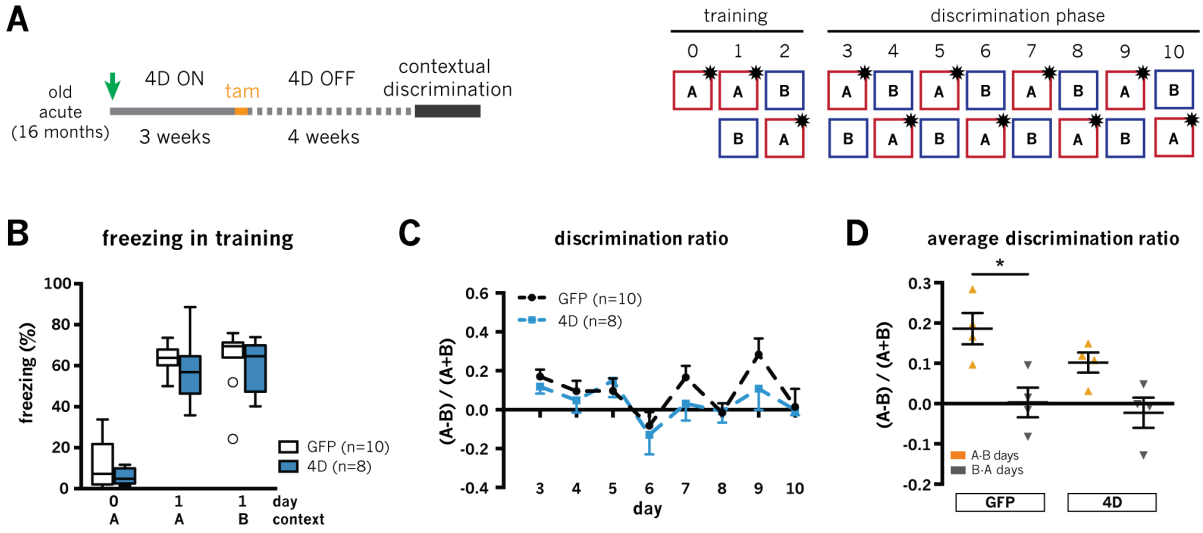

Fig. 3.15: An acute increase in neurogenesis reduces the bias for procedural learning in a fear contextual discrimination task.

A - D. Experimental layout and behavioural paradigm (**A**), freezing during training (**B**), discrimination performance during the test (**C**) and in days with A-B or B-A presentation order (**D**) of GFP vs. 4D injected old mice (16 months old; n = 10 and 8, respectively) tested 4 weeks after tamoxifen administration in my contextual discrimination paradigm. Boxplots represent freezing per context and day as indicated (B). Values represent means of each day (C) or group of days (D). * $p < 0.05$.

In essence, an acute increase in adult neurogenesis in 18 months old mice was by itself sufficient to reverse the age-related switch from contextual to procedural learning rescuing allocentric navigational strategies and memory and, to a lesser degree, discrimination. As the latter task might have been too difficult for such an advanced age and since mice must have lost contextual discrimination at some point between youth and senescence, I next attempted to delay this impairment by a chronic expansion of NSC throughout life.

A chronic increase in neurogenesis preserves contextual learning and memory throughout life

Young mice (4 months old) were injected with GFP or 4D viruses without administration of tamoxifen and tested for contextual discrimination at middle age (**Fig. 3.16A**). After one day of training both cohorts showed similar learning and generalization in context A and B (**Fig. 3.16B**). Interestingly, 7 months old GFP-injected mice already showed a pattern of discrimination that was similar to that of 16-18 months old mice with positive discrimination in A-B, but not B-A, days (0.30 ± 0.12 vs. 0.00 ± 0.09, respectively; $p = 0.014$; **Fig. 3.16C**) indicating that contextual discrimination was already impaired at this age. In contrast, 4D-treated animals preserved the ability to discriminate contexts in consecutive days throughout the test with no significant difference between A-B and B-A days (0.27 ± 0.08 vs. 0.16 ± 0.08, respectively; $p = 0.14$; **Fig. 3.16D** and **E**), showing that an increased neurogenic state is sufficient to preserve contextual discrimination during aging.

Memory was tested 2 months later in 9 months old mice in which both control and 4D groups showed increased freezing in A when presented in the A-B order (A vs. B: 32.5 ± 5.3 vs. 5.8 ± 1.5% and 33.9 ± 5.1 vs. 15.2 ± 3.8%, respectively; both $p < 0.005$) but only 4D-treated mice kept this trend the day after in the B-A order (A vs. B: 14.9 ± 3.5 vs. 9.6 ± 2.1% and 18.8 ± 3.8 vs. 10.1 ± 3.1%; $p = 0.23$ and 0.051; respectively; **Fig. 3.16F**). One additional probe trial 2 weeks later showed that controls had completely lost contextual conditioning irrespective of the presentation order while 4D-treated mice still maintained positive levels of discrimination in the A-B order (A vs. B: 5.3 ± 1.2 vs. 2.9 ± 0.8% and 8.1 ± 2.4 vs. 2.8 ± 1.1%; $p = 0.21$ and 0.026, respectively; **Fig. 3.16G**). Finally, the same cohorts of mice were tested for reacquisition of memory by a second training at 12 months. After the first day of re-training, controls did not show any

discrimination between context A or B (20.3 ± 10.1 vs. 23.3 ± 14.1%; $p = 0.62$) suggesting a complete loss of the initial memory, while 4D mice showed not only more freezing in A relative to B (35.5 ± 15.7 vs. 22.2 ± 15.8%; $p = 0.030$) but also more freezing in A relative to controls (35.5 ± 15.7 vs. 20.3 ± 10.1%, respectively; $p = 0.029$; **Fig. 3.16H**), suggesting an efficient recovery of the memory.

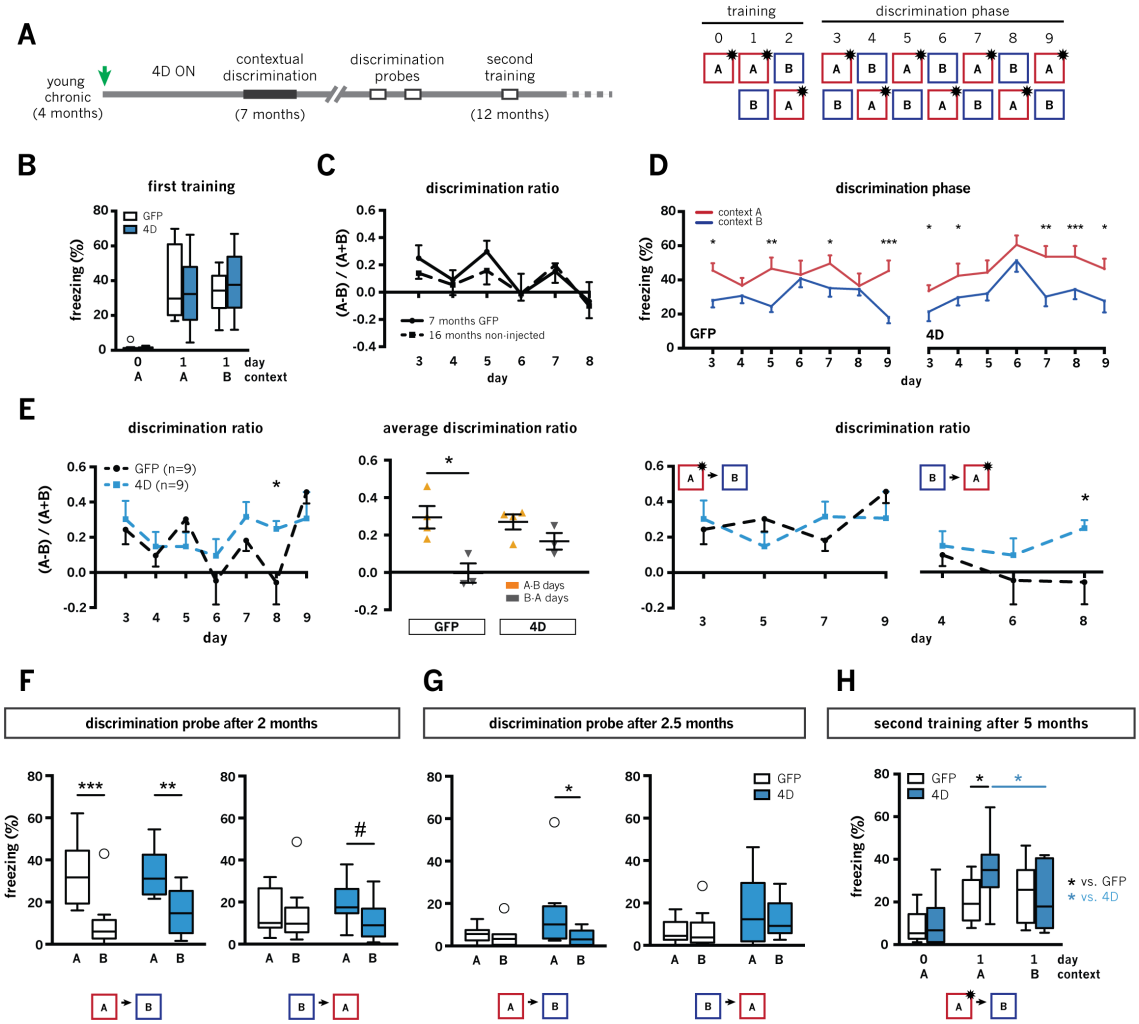

Fig. 3.16: A chronic increase in neurogenesis preserves contextual discrimination during aging.

A - E. Experimental layout and behavioural paradigm (**A**), freezing during training (**B**) and discrimination phase (**D**) and discrimination performance during the test and in days with A-B or B-A presentation order (**E**) of GFP vs. 4D injected old mice (16 months old; n = 9 and 9, respectively) tested 3 months after viral infection in contextual discrimination. **C**. Comparison of performance between 7 months old chronic GFP-injected and 16 months old untreated mice (from 3.9C). **F - H**. Memory discrimination probes at 2 and 2.5 months (**F** and **G**) and second training at 5 months after the test (**H**). Boxplots represent freezing per context and day as indicated. Values represent means of each day (C, D and E, left) or group of days (E, middle). * $p < 0.05$. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

The same cohort of mice was tested 2 months later in the Barnes maze to assess the impact of chronic 4D-increase in neurogenesis on navigational tasks (**Fig. 3.17A**). Results showed that 14 months old control mice preserved some degree of contextual navigation during learning that was almost completely replaced by procedural navigation after reversal (**Fig. 3.17B**), pointing out specific impairments in flexible learning. Increased neurogenesis compensated this effect by favouring contextual strategies at the expense of procedural ones after reversal (OR = 2.76; $p = 0.008$ and OR = 0.33; $p < 0.001$, respectively; **Fig. 3.17B**). When mice were tested for memory 6 weeks later, only 4D-treated mice showed spatial preference for the last position of the escape box (17.05 ± 12.93 vs. 34.16 ± 2.69%, respectively; $p = 0.036$; **Fig. 3.17C**), revealing a more solid place memory.

Next, I investigated whether increased neurogenesis influenced memory engram formation. Given that the adoption of contextual versus procedural learning was proposed to depend on hippocampal versus striatal function (Bach et al., 1999; Hartley et al., 2003; Iaria et al., 2003; Packard and McGaugh, 1996), neuronal activity in these areas was assessed by c-Fos quantification immediately after the memory trial. In the DG of 4D-treated mice, the proportion of c-Fos+/GFP+ cells was decreased relative to controls (1.84 ± 0.41 vs. 2.97 ± 0.68%, respectively; $p = 0.040$; **Fig. 3.17D**). Moreover, this led to a different activation ratio of c-Fos+ cells in the hippocampus relative to striatum in 4D compared to control mice (0.79 ± 0.12 vs. 1.04 ± 0.03, respectively; $p = 0.019$; **Fig. 3.17D**), in line with the differential use of contextual vs. procedural learning and memory between groups. Taken together, these data show that a sustained increase in hippocampal neurogenesis preserved contextual learning and memory circuits and improved cognition throughout life.

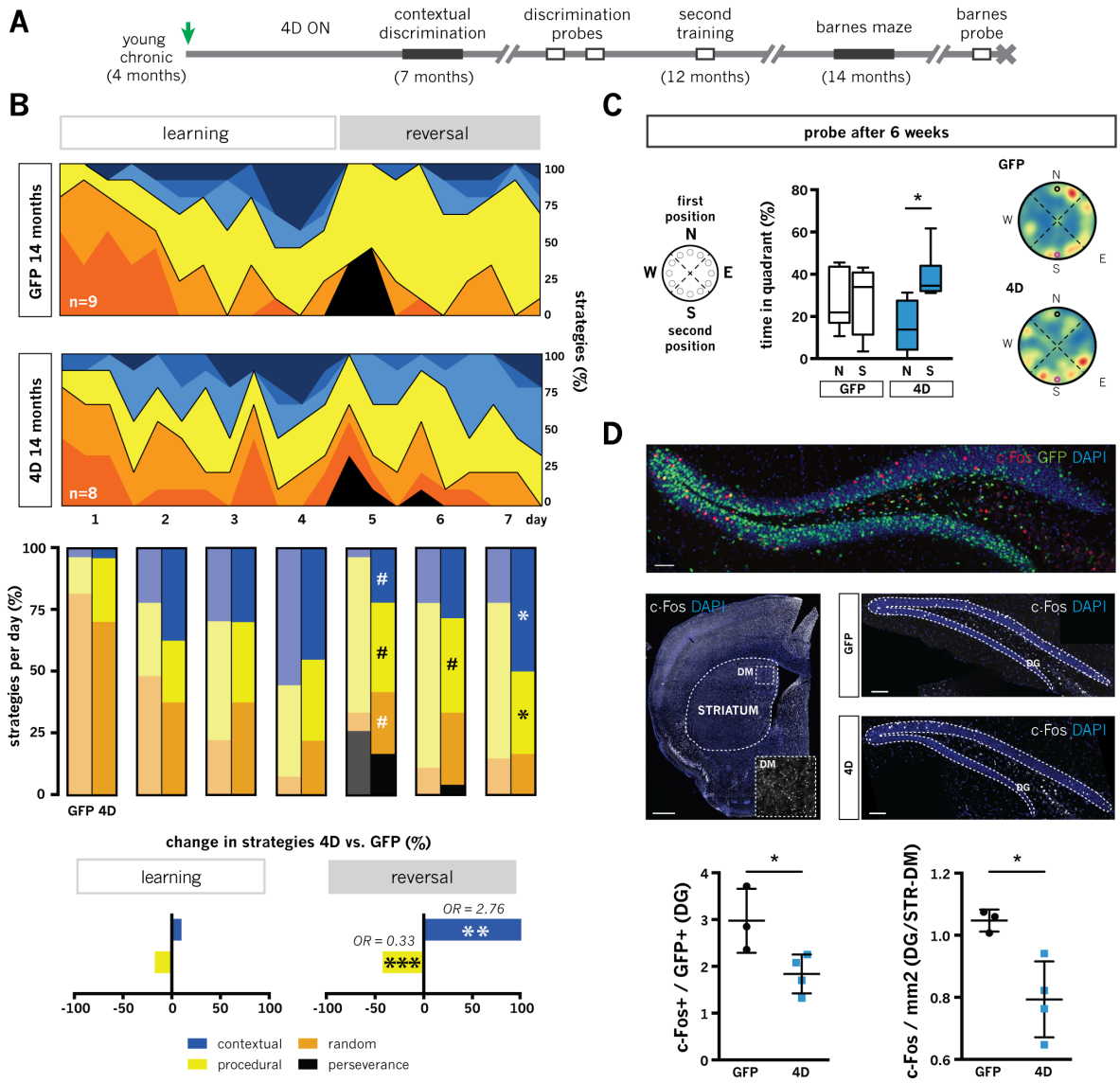

Fig. 3.17: A chronic increase in neurogenesis preserves spatial navigation throughout life.

A - C. Experimental layout (**A**), learning strategies contribution per group, day and percentage change (**B**) and memory probe trial with heat maps (**C**). **D**. c-Fos activation pattern after the probe test in the striatum and DG and quantifications. Boxplots represent percentage time per quadrant (C) and values represent proportion of cells positive for c-Fos within GFP+ or per brain structure area in GFP and 4D mice, as indicated (mean ± sd; n = 3 and 4 respectively). Scale bars = 100 µm (DG sections) and 250 µm (striatum section). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

CHAPTER 4

DISCUSSION

In this work, I studied the impact of NSC expansion on cognition throughout life, showing that increased neurogenesis can be used as a rejuvenating approach to ameliorate age-related decay in hippocampal function. Some of these results are worth discussing.

4.1 CONSIDERATIONS ABOUT THE 4D MODEL

Most of the approaches aiming to manipulate neurogenesis to study its role lack specificity. Meta-analysis of published results on neurogenesis ablation studies revealed high levels of heterogeneity among experiments and contradictory findings (Groves et al., 2013). On the other hand, most of the gain-of-functions methods such as running or enriched environment have interfering secondary effects (van Praag, 2009; van Praag et al., 2000). All these experiments contributed to our understanding of the function of neurogenesis, but more

specific tools are needed to further elucidate the contribution of newborn neurons to hippocampal function.

The 4D model could significantly help in this endeavour. 4D effect is transient and restricted to the NSC expansion phase, influencing neurogenesis only in its first step. Interestingly, recombination of the 4D genes immediately after viral injection does not result in increased neurogenesis, proving that the effect of 4D is cell-intrinsic and dependent on NSC expansion (Artegiani et al., 2011). After recombination, physiological differentiation results in a 2-fold increase in neurogenesis that has been consistently observed in all the experiments in this work. Importantly, the NSC neural progeny no longer overexpress 4D, and it is morphologically undistinguishable from physiologically generated cells. It is likely that the only difference between GFP controls and 4D injected brain is the number of newborn neurons integrated into the circuit, although a full electrophysiological study would be necessary to support this conclusion. This makes a difference with previous genetic gain-of-function models that artificially affect the competition between newborn and mature granule cells, interfering with the previously established circuit (Adlaf et al., 2017; McAvoy et al., 2016; Ryu et al., 2016; Sahay et al., 2011a). An additional advantage of the 4D system is its resolution. Tamoxifen acts as a synchronizer, releasing the brake of differentiation and driving the generation of the first wave of increased neurogenesis simultaneously. This allows the study of the influence of a single expanded cohort of age-matched neurons at different stages of maturation which, as shown in this work, is important given the shift in electrophysiological properties of adult-generated neurons.

More molecular studies are needed to fully understand the mechanisms by which NSC expand and differentiate after 4D manipulation. For instance, it was believed that the main force driving differentiation was the increased number of NSCs

after the expansion phase (Artegiani et al., 2011). Yet, the presence of more NSC 2 weeks after tamoxifen and absence of later waves of neurogenesis in the infected population suggest other valid explanations. Neurogenesis levels could be determined by the demand of neurons in the DG. Rather than the excess of NSCs, the transient decrease in neurogenesis during the expansion phase might generate an accumulating neuronal need that can only be satisfied by the NSCs after recombination. Previous knowledge supports this hypothesis. Neuronal demand leads to changes in the general activity of the DG, given the power of newborn neurons to control DG excitability (Drew et al., 2016; Ikrar et al., 2013; McAvoy et al., 2015; Ming and Song, 2011; Sahay et al., 2011b). These changes in circuit activity have been proposed to be sensed by parvalbumin GABA interneurons, which in turn can regulate neurogenesis as a niche mechanism to sense and adapt to changes in processing requirements (Dranovsky et al., 2011; Song et al., 2012, 2013). Hence, during the expansion phase a transient decrease in neurogenesis could recruit less GABA inhibition (Drew et al., 2016; Temprana et al., 2015), instructing more NSC to differentiate as soon as 4D is shut off. Once the increased wave of neurogenesis is generated, the resulting increase in GABA would arrest NSC activity (Song et al., 2012), recovering basal levels of neurogenesis in the infected area. Additionally, this increase in GABA signalling could also participate in spatial homeostasis and promote neuroblast survival in the less-infected area (Song et al., 2013), leading to the local extrinsic effect observed in this experiment (**Fig. 4.1**). Such regulatory mechanism would explain the presence of transient waves of neurogenesis in temporally-controlled genetic models, even locally in adjacent regions of the same section (as also seen in McAvoy et al., 2016). Furthermore, the 2-fold increase in neurogenesis consistently found in this work could represent an homeostatic limit of such regulatory mechanism, present in different conditions (acute vs. chronic) and ages.

In summary, the 4D model provides an useful tool to investigate not only the effect of increased neurogenesis in cognitive function, given its specificity and resolution, but also the molecular mechanisms that lead to the temporal and spatial homeostatic regulation of the neurogenic process itself.

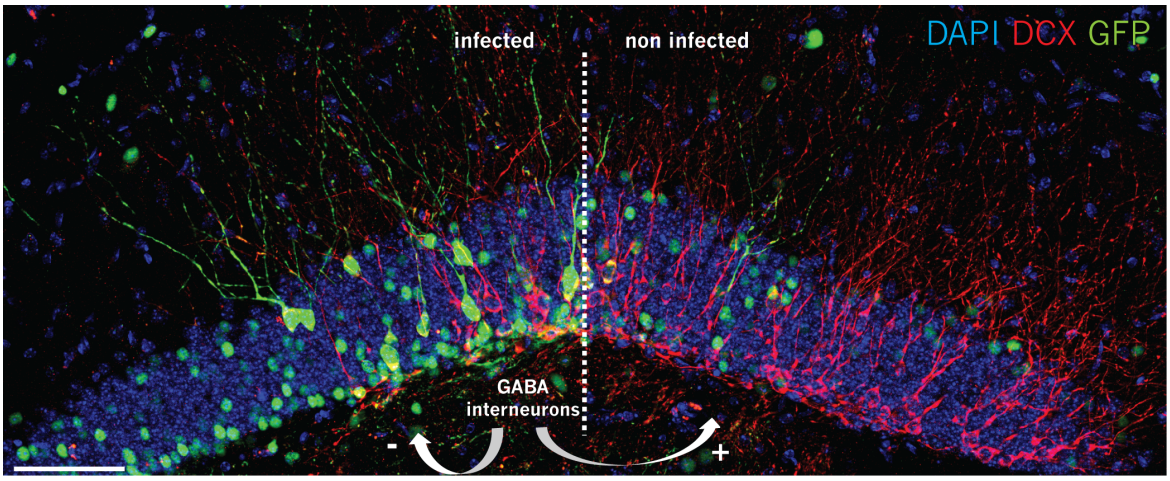

Fig. 4.1: Homeostatic regulation of neurogenesis in differentially infected DG compartments.
Circuit activity is highly sensitive to changes in neurogenesis (Drew et al., 2016; Ikrar et al., 2013; McAvoy et al., 2015; Ming and Song, 2011; Sahay et al., 2011b). GABA interneurons can sense those changes in activity, and couple activity demand to neurogenic production through the release of GABA influencing both NSC proliferation and neuroblast survival (Song et al., 2013, 2012). Thus, after the doubling in neurogenesis generated by 4D, local GABA interneurons could be mediating the return to basal levels of neurogenesis in the infected area together with an increase in neuroblast survival in the non-infected area. The picture corresponds to a magnification of 4D-infected brain in the **Fig. 3.5B.**

4.2 THE NATURE OF FLEXIBILITY

Evaluation of the influence of a single, expanded cohort of newborn neurons revealed improvements in reversal learning in young mice tested in the water maze. Interestingly, these improvements were specific to 4 weeks old neurons and disappeared at later time points, consistent with the physiological loss of plastic electrophysiological properties of these cells from 6 weeks onwards (Espósito et al., 2005; Gu et al., 2012a; Schmidt-Hieber et al., 2004; Snyder et al., 2001).

The 4D model adds a new level of specificity and resolution to early hypotheses suggesting a role of newborn neurons in decreasing memory interference and promoting flexible learning (Garthe et al., 2016; McAvoy et al., 2016; Wang et al., 2014; Wiskott et al., 2006). Cognitive flexibility is often defined as the adaptation to changes in environmental contingencies or, applied to this experiment, successful learning upon reversal in the water maze. Interestingly, in this and other paradigms the manipulation of neurogenesis seems to have a bigger impact on reversal and not during the first learning phase (Burghardt et al., 2012; Garthe et al., 2009; Swan et al., 2014).

It has been recently proposed that one neurogenic-dependent mechanism for such flexibility is the clearance of the first conflicting memory, leading to less interference with the learning of the second condition (Anacker and Hen, 2017; Epp et al., 2016). However, 4D-mice with improved reversal learning showed preference for the first platform position in the probe test 2 weeks after the water maze, revealing for the first time that mice with increased neurogenesis flexibly learnt faster during reversal without interfering with the first stronger memory. Furthermore, control mice showed no preference during the probe test suggesting interfering re-learning or forgetting. It seems therefore that, rather than promoting the clearance of the first memory engram, newborn neurons helped in

its consolidation while facilitating the non-overlapping learning of a similar memory, suggesting improved pattern separation function. Indeed, reversal can be understood as a pattern separation task in that learning of the second condition must be separated from the first one. Accordingly, reversal and flexible learning have been argued to be a manifestation of improved spatial memory and contextualization (Garthe and Kempermann, 2013; Gonçalves et al., 2016).

The neuronal correlates of cognitive flexibility have hardly been investigated. Immediate early genes (IEGs) like Arc or c-Fos, that are shortly expressed after neuronal activation, are broadly used as reliable indicators of activity and memory trace ensemble organization (Minatohara et al., 2016). A previous study reported an increase in DG activity (Arc+ cells) in mice with impaired flexible learning upon neurogenesis ablation (Burghardt et al., 2012), which is in line with the proposed function of neurogenesis in regulating DG activation through feed-back inhibition (Drew et al., 2016; Ikrar et al., 2013) and ultimately controlling sparseness and pattern separation function (McAvoy et al., 2015; Sahay et al., 2011b). This finding seems at odds with the increase in Arc+ cells in 4D-mice with more neurons and improved flexible learning (**Fig. 3.6E**). The increase in activity after neurogenesis arrest reported by Burghardt et al. (2012) was suggested to interfere with the segregation between conflicting memories, leading to impaired flexibility. I propose that the increase in Arc+ cells in 4D brains might reflect the cellular processing needed to learn something new without interfering with an old, similar memory. Relational representations are characterized by the flexible recombination and update of its components (Rubin et al., 2014). It seems intuitive to think that in reversal learning tasks in which disambiguation between a new and old condition must be learnt, and taking into account that the first memory is retained, it might be necessary to relate both memories at the same time (in my recurrent example, the ability to remember your car's location in the parking lot yesterday and simultaneously distinguishing

it from the location the day before). The increase in Arc+ cells in mice learning more flexibly could be underlying this parallel retrieval, or at least an increased computational effort to keep the first memory while acquiring the second one. More experiments, especially the use of the recently developed engram technology allowing the tagging of specific memories (Ramirez et al., 2013; Tonegawa et al., 2015), could significantly contribute to clarify the role of neurogenesis in this and other mnemonic processes (Anacker et al., 2015). For the moment, these findings could help in the understanding of the mechanisms of flexible learning, the difference with re-learning and how newborn neurons participate in the reduction of memory interference to promote cognitive flexibility.

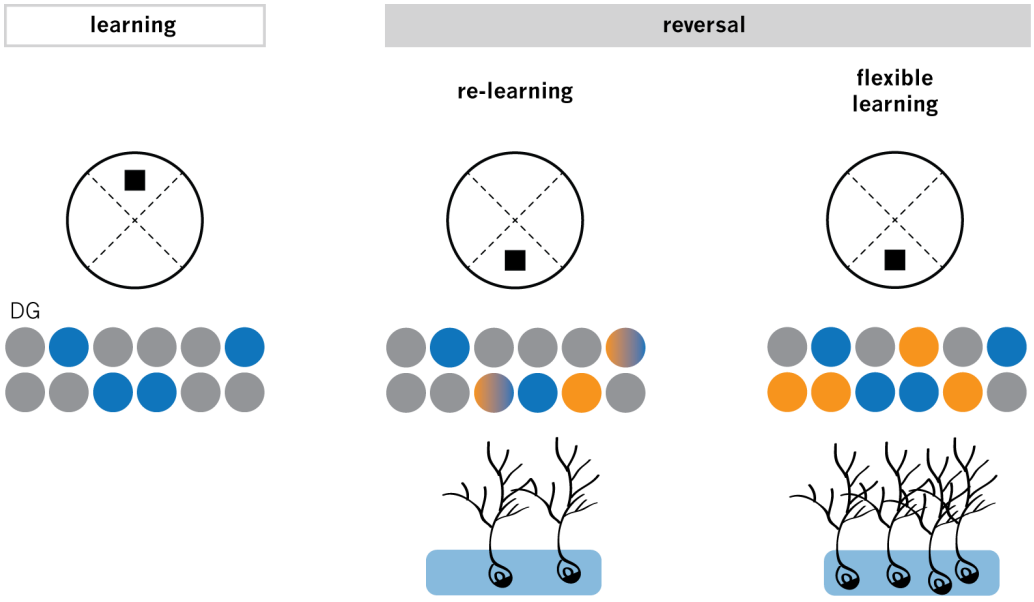

Fig. 4.2: Flexible learning and re-learning.

Cartoon depicting a possible mechanism for flexible learning. Low levels of neurogenesis could lead to memory interference by neuronal ensemble overlapping (first and second memories engrams, in blue and orange respectively). As a consequence, control mice showed no preference in the probe memory test for either position of the platform. Alternatively, increased levels of neurogenesis lead to the separation and conservation of both memory engrams, allowing a faster reversal learning. In the probe test, these mice showed preference for the stronger first memory, demonstrating that reversal learning was not interfering (i.e., was flexible). An increased DG activity during reversal learning could underscore the neuronal substrate of parallel processing and separation of both memories.

4.3 REJUVENATING THE BRAIN

The results presented in this work show a cumulative decay in hippocampal function and neurogenesis throughout life, as also shown in a large body of literature. The combination of two different hippocampal-dependent tasks revealed the evolution of these cognitive impairments, from an early loss of contextual discrimination at middle-age, to defects in navigational tasks first affecting reversal learning and later on general, spatial learning. Moreover, this decrease in hippocampal function is accompanied by an increase in the use of procedural, striatal learning widely reported in the literature (Bach et al., 1999; Barnes et al., 1980; Bohbot et al., 2012; Head and Isom, 2010; Leal and Yassa, 2015; Lester et al., 2017). Several reasons, ranging from molecular to circuit modifications have been proposed to explain this age-related hippocampal decay, being a decrease in neurogenesis numbers only one of them (Leal and Yassa, 2015; Lester et al., 2017; Samson and Barnes, 2013). While it is unclear whether the decline in contextual, hippocampal learning parallels that of neurogenesis (Bizon and Gallagher, 2003; Drapeau et al., 2003; Gil-Mohapel et al., 2013), it is remarkable that all these impairments were compensated or prevented by 4D-increase in neurogenesis throughout life.

Previous studies reported beneficial effects of increased neurogenesis on cognition during aging (McAvoy et al., 2016; Montaron et al., 2006; Seib et al., 2013; van Praag et al., 2005; Villeda et al., 2011; Wu et al., 2015). Yet, my work is the first that relates those improvements to the adoption of more flexible, hippocampal dependent learning strategies.

Assessment of the learning strategies is essential. Commonly used parameters, such as latency or freezing, are mere behavioural readouts insufficient to dissect the brain process used during learning (Garthe and Kempermann, 2013), and can

actually be misleading. This can be seen in the Barnes maze, when chaining led to constant latencies after reversal suggesting impairments in animals with more hippocampal learning (**Fig. 3.14B),** as well as in fear conditioning, when similar freezing levels resulted in different brain activity patterns in young vs. old mice (**Fig. 3.10**). Alternatively, assessment of the strategies gives valuable information. It underscores the memory system engaged during learning (Squire, 2004), the nature of the memory created (place vs. response) and intrinsic features of contextual and procedural learning themselves. For example, while procedural strategies contribution hardly changed in control mice before and after reversal in the Barnes maze, contextual strategies consistently needed to be re-acquired as a consequence of the update of the allocentric map, until they reached comparable levels with the first acquisition phase. In fact, this probably led to preferences for the second position in the Barnes maze probe tests, in contrast with the water maze results. Importantly, evaluation of the learning strategies also discriminated between memory systems usage irrespective of whether the test could be successfully solved or not by using a given strategy (procedural learning in the Barnes maze vs. contextual discrimination). The only studies selectively increasing neurogenesis and improving cognition during aging (McAvoy et al., 2016; Seib et al., 2013) primarily assessed memory persistence without investigating whether this was caused by the adoption of strategies that might be intrinsically easier to remember. In contrast, my work emphasizes the importance of learning strategy usage during aging rather than the efficacy of the strategies themselves, highlighting the rejuvenating potential of adult neurogenesis in the recovery and preservation of flexible, contextual memory systems during aging.

Interestingly, the number of newborn hippocampal neurons calculated in 18 months old mice changed from ca. 70 to 180 upon acute 4D manipulation representing an increase by about 100 neurons in the whole brain (not shown). This is remarkable because the levels of neurogenesis in 4D-treated, old mice were

a fraction of those physiologically generated in young mice, yet enough to rejuvenate hippocampal function, navigational strategies and memory. These findings suggest that early interventions that rescue cell function even to levels otherwise considered negligible may still provide functional benefits. These findings well reconcile with the neural reserve hypothesis (Kempermann, 2008) showing that such reserve can be extrinsically exploited during aging or potentially disease, and providing a proof-of-principle relevant in facing the challenges of an aging population.

4.4 FUTURE OUTLOOK

On a broader perspective, and combining the results obtained during youth and senescence, increased neurogenesis upon 4D manipulation helped in the flexible relation of spatial and contextual information, a function long been attributed to hippocampal circuits (Eichenbaum, 2000, 2017a; Eichenbaum and Cohen, 2014; Rubin et al., 2014; Squire, 2004). However, given the lack of mechanistic experiments, any theory about how the 4D-increase in neurogenesis contributes to hippocampal function and rejuvenation across the lifespan is purely speculative.

In fact, how increased neurogenesis achieved by any means translates to changes into the electrophysiological parameters ultimately governing memory function remains largely unknown. Given the power of 4D in cognition enhancement, its specificity and resolution, the resultant increase in neurogenesis could provide the perfect framework to study the circuit consequences of increased neurogenesis. One example of such experiments could include single-units recordings in aged control and 4D mice, searching for changes in the hippocampal place cells physiology used for navigation (an experiment only modelled so far, Cuneo et al., 2012). Other assessments could include sequential reactivation during sleep and LTP measurements, both important processes for the consolidation of acquired memories that has been shown to be disrupted during aging (Barnes, 1979; Barnes et al., 2000; Gerrard et al., 2008; Smith et al., 2000; Wiegand et al., 2016).

New technologies are being currently develop that allow for the identification of the neuronal substrates of memory formation as well as their specific manipulation (Ramirez et al., 2013; Tonegawa et al., 2015). Combination if this technology with the 4D model could help to shed light in the discussion of whether newborn neurons carry the memory message or dictate the tone of the DG (Piatti et al., 2013). For instance, one could analyse memory engram formation in different

conditions: more or less neurogenesis in young and old animals, activation vs. inhibition of 4D-generated neurons in different maturational stages or different moments of behaviour, assessing their specific role in acquisition vs. retrieval... This represents a whole new dimension in the perspectives of neurogenesis research, and combination of these approaches could help to answer questions that have long been formulated.

Not only this is important for neurogenesis field, but also for neuroscience in general. A deeper understanding on the circuit consequences of the 4D rejuvenating effect may lead to new discoveries with regard to brain processing and efficient learning throughout life. Implementation of more clinically-oriented experiments exploiting this knowledge could move forward the fields of aging and neurodegeneration. Finally, these potential findings might give us the opportunity to replicate the functional gain of increased neurogenesis by artificial means such as deep-brain-stimulation or implanted electrodes.

Altogether, the findings presented in this thesis and the exciting perspectives of the experiments yet to come will contribute to our understanding of the role of adult hippocampal neurogenesis, its influence in memory systems and ultimately its potential in rejuvenating brain function during aging.

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APPENDIX I

Technische Universität Dresden

Medizinische Fakultät Carl Gustav Carus

Promotionsordnung vom 24. Juli 2011

Erklärungen zur Eröffnung des Promotionsverfahrens

1. Hiermit versichere ich, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; die aus fremden Quellen direkt oder indirekt übernommenen Gedanken sind als solche kenntlich gemacht.
2. Bei der Auswahl und Auswertung des Materials sowie bei der Herstellung des Manuskripts habe ich Unterstützungsleistungen von folgenden Personen erhalten: nicht zutreffend.
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4. Die Arbeit wurde bisher weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.
5. Die Inhalte dieser Dissertation wurden in folgender Form veröffentlicht: nicht zutreffend
6. Ich bestätige, dass es keine zurückliegenden erfolglosen Promotionsverfahren gab.
7. Ich bestätige, dass ich die Promotionsordnung der Medizinischen Fakultät der Technischen Universität Dresden anerkenne.
8. Ich habe die Zitierrichtlinien für Dissertationen an der Medizinischen Fakultät der Technischen Universität Dresden zur Kenntnis genommen und befolgt.

Dresden, den Gabriel Berdugo-Vega

APPENDIX II

Hiermit bestätige ich die Einhaltung der folgenden aktuellen gesetzlichen Vorgaben im Rahmen meiner Dissertation

- das zustimmende Votum der Ethikkommission bei Klinischen Studien, epidemiologischen Untersuchungen mit Personenbezug oder Sachverhalten, die das Medizinproduktegesetz betreffen
  Aktenzeichen der zuständigen Ethikkommission
  DD 24-9168.11-1/2011-11, HD 35-9185.81/G-61/15
- die Einhaltung der Bestimmungen des Tierschutzgesetzes
  Aktenzeichen der Genehmigungsbehörde zum Vorhaben/zur Mitwirkung
  TVV 13/2016
- die Einhaltung des Gentechnikgesetzes
  Projektnummer
  DD 24-9168.11-1/2011-11
- die Einhaltung von Datenschutzbestimmungen der Medizinischen Fakultät und des Universitätsklinikums Carl Gustav Carus.

Dresden, den Gabriel Berdugo-Vega