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Individual performance in cognitive aging and deficit prevention by cognitive training in the rat: integration of structural, molecular and functional correlates

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e do
Professor Doutor João Carlos Sousa

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Performance individual no envelhecimento cognitivo e prevenção de défices pelo treino cognitivo no rato: integração de correlatos estruturais, moleculares e funcionais

Resumo

O envelhecimento está associado a défices de memória. No entanto, as alterações cognitivas relacionadas com o envelhecimento variam significativamente entre indivíduos e domínios cognitivos. Até ao momento, a evidência sobre os mecanismos por detrás desta heterogeneidade é diminuta.

Neste trabalho, fizemos a caracterização comportamental de uma grande coorte de ratos envelhecidos e jovens adultos. Mostramos que os ratos envelhecidos apresentavam uma menor aprendizagem espacial e flexibilidade comportamental. De realçar, o grau de declínio cognitivo foi altamente variável. Dada esta variabilidade, agrupamos os animais envelhecidos e jovens de acordo com a sua performance, caracterizando-os como bons ou maus. De seguida, exploramos os correlatos estruturais, funcionais e moleculares destas diferenças comportamentais no hipocampo e córtex pré-frontal medial, regiões chave envolvidas no funcionamento cognitivo e particularmente vulneráveis ao processo de envelhecimento. Os resultados mostraram que as diferenças individuais da função cognitiva podem ser correlacionadas com alterações estruturais, funcionais e moleculares nestas regiões, no entanto em sentidos opostos, tratando-se de animais novos ou envelhecidos. Enquanto que para os animais novos "maior é melhor", parece que "menor é melhor" é mais apropriado aos animais envelhecidos. Nestes, a marcada heterogeneidade comportamental poderá ser atribuída a variações nos níveis de neurotrofinas e a um decréscimo da atividade autofágica.

Pelo descrito, o desenvolvimento de intervenções que otimizam o funcionamento cognitivo é da maior relevância. Foi já descrito que o treino cognitivo pode aumentar a memória. Por esta razão, ensaiamos se o teste da tábua perfurada seria capaz de prevenir défices cognitivos dependentes de memória. As nossas descobertas sugerem que este treino tem efeitos benéficos duradouros.

Potencialmente, o trabalho aqui apresentado poderá contribuir para a compreensão das diferenças cognitivas individuais relacionadas com a idade, podendo auxiliar no desenvolvimento de novas vias terapêuticas para um envelhecimento cerebral saudável.

Palavras-chave: alterações estruturais, autofagia, défices cognitivos, envelhecimento, heterogeneidade.

Individual performance in cognitive aging and deficit prevention by cognitive training in the rat: integration of structural, molecular and functional correlates

Abstract

Aging is commonly associated with memory impairments. However, age-related changes vary considerably across individuals and cognitive domains. Thus far, the evidence on the mechanisms underlying such heterogeneity is scarce.

Thus, in the present work, we behaviorally characterized a very large cohort of old age and young adult male rats. We showed that old rats, on average, had poorer spatial learning and behavioral flexibility than young adults. Of notice, the degree of cognitive decline was highly variable. Given this variability, we clustered young and old animals according to individual cognitive performance and classified them as good and bad performers. We then explored the structural, functional, and molecular correlates of such behavioral differences in the hippocampus and medial prefrontal cortex. Both areas are key regions involved in cognitive functioning and particularly vulnerable to the aging process. The results of our work showed that individual differences in cognitive function can be correlated with structural, functional, and molecular changes in these brain regions, albeit in different directions in young and old animals. While for young individuals "bigger is better", it seems that "smaller is better" is a more appropriate aphorism in what regards old subjects. Moreover, we provided evidence that, in older animals, dendritic length and volumetric differences, and concomitant behavioral heterogeneity, can be ascribed to variations in neurotrophin levels and decreased autophagic activity.

In line with these results, the development of interventions to maintain cognitive functioning is of utmost importance. It's known that cognitive training can enhance memory function. Therefore, we tested if the Hole Board test was able to prevent age-associated deficits. Our findings suggested that cognitive training had long-lasting improvements in age-induced impairments.

Hopefully, the work we herein present will contribute to the understanding of age-related individual differences, both in rodents as in humans. We hope our work will aid in the development of new therapeutic avenues towards a successful brain aging and an increased "mindspan".

Keywords: aging, autophagy , cognitive impairments, heterogeneity, structural correlates.

Table of contents

List of abbreviations

AD – Alzheimer's disease

BDNF – Brain-derived neurotrophic factor

BPs – Bad performers

CA – Cornu Ammonis

Cg – Cingulate area

CNS – Central nervous system

DG – Dentate gyrus

DHPC – Dorsal hippocampus

EC – Entorhinal cortex

EE – Environmental enrichment

GPs – Good performers

HB – Hole Board

HPC - Hippocampus

IL – Infralimbic area

LTP – Long-term potentiation

MO – Medial orbital area

mPFC – Medial prefrontal cortex

mTOR – Mechanistic target of rapamycin

MWM – Morris water maze

OFC – Orbitofrontal cortex

PD – Parkinson's disease

PFC – Prefrontal cortex

PL – Prelimbic area

PSD95 – Postsynaptic density protein 95

Sb – Subiculum

SNAP25 – Synaptosomal-associated protein 25

SYP – Synaptophysin

TM – T-maze

VHPC – Ventral hippocampus

Thesis planning

The present thesis is organized into five different chapters. Chapter I consists of the introduction and chapter V of the general discussion, conclusions and future perspectives. In chapters II to IV, the major findings of this thesis are presented in the format of research papers.

Chapter I provides a brief overview of the central research theme, focusing on the healthy aging brain and its heterogeneity. This is followed by a description of the several age-related alterations (structural, functional, molecular) seen in aging brains that may explain age-associated cognitive decline. Lastly, the role of cognitive training in healthy aging will be discussed. In this first chapter, we also address the major questions focused on this thesis.

Chapter II consists of the paper entitled "Structural and molecular correlates of cognitive aging in the rat", accepted for publication in the journal *Scientific Reports*. In this work, we showed that old rats, on average, present poorer spatial learning and behavioral flexibility than young adults, with an emphasis given to the highly variable degree of cognitive decline. Using 3-dimensional neuronal reconstructions and western blot analysis, we were able to explore the structural and molecular correlates of such behavioral differences. Thus, in this experimental work, we provided evidence that dendritic length alterations in the hippocampus (HPC) and medial prefrontal cortex (mPFC), concomitant to behavioral performance heterogeneity of older animals, can be ascribed to variations in neurotrophin levels and, more importantly, autophagic activity, leading to dendritic pruning deficits in the cognitively deprived old animals.

In **Chapter III** the experimental work: "Bigger is worse: volumetric correlates of age-related changes in hippocampus and medial prefrontal cortex in the rat", is an extension of the work discussed in chapter II. Together with our previous findings relating longer dendritic trees and autophagy deficits to cognitive deficits in the older population, the results presented in this chapter suggest that volumetric alterations are associated with age-related HPC- and mPFC-dependent behavior deficits.

Chapter IV, "Adulthood cognitive training selectively prevents aged-related memory impairments in the rat", focuses on the impact of cognitive training in preventing or promoting recovery from age-related cognitive decline, while acknowledging the well-described heterogeneity of brain aging. In this assessment, we show that cognitive training has long-lasting consequences by enhancing brain plasticity mainly within the brain circuits involved in the specific training task, through learning-related processes of neurite architectural remodeling. Importantly, this study suggests that even a relatively short period of

HPC-dependent cognitive training is sufficient to improve the ability to recover from age-induced HPC-dependent impairments, which might prove relevant for healthy aging.

In **Chapter V** we present a general discussion and conclusions of this thesis, as well as the future perspectives that are envised from this experimental work.

Chapter I

Introduction

1. General considerations

Aging is an inevitable biological process characterized by a declining ability to respond to stress, increasing homeostatic imbalance and increased risk of disease eventually ending in death (Tosato *et al.*, 2007).

Nowadays, worldwide, the number of individuals aged over 65 years old is growing faster than any other age group (Lister & Barnes, 2009). As a consequence of cumulative challenges over the lifespan, the majority of these individuals have to deal with alterations in their bodies, including the brain. Thus, this increase in life expectancy has revealed a new "epidemic": the elderly are at significant risk for dementia, a syndrome characterized by impaired memory and cognitive abilities, affecting their functional independence. Although modern medicine has allowed fixing or replacing many functions on our bodies, our brain is highly customized, and pathological alterations are often irreversible. Hence, for the majority of aged individuals which are in good physical condition, cognitive decline is the main threat to their quality of life. Because of this growing threat, and in line with the recognition that aging is not a disease in itself, but instead comprises natural biological processes that are, nevertheless, amenable to experimental study, the question of the effect of aging upon the brain has received increased attention within the recent years, and the promotion of "mindspan" (the maintenance of mental abilities over the lifespan) has become a top priority (Gallagher *et al.*, 2011).

The development of rational approaches to diagnose and treat unhealthy aging is, therefore, of paramount importance. Accordingly, a thorough understanding of the multiple mechanisms underlying brain aging is of utmost interest.

In recent years, researchers have made rapid progress in understanding the neural changes that affect the life course of cognitive capabilities. New animal models have been developed and old ones have become better characterized and standardized (Mitchell *et al.*, 2015). In addition, advances in brain imaging techniques now permit investigations in aged humans with amazing resolution and sophistication, providing a bridge between human and animal studies. Altogether, these advances will be useful to develop behavioral and technological interventions aiming to maintain cognitive performance in older individuals.

Despite all the advances made in the field, one of the main questions still to be answered is the underlying mechanisms that allow some individuals to age without appreciable cognitive decline while others show major losses of cognitive functions. A vast majority of the existing theories report that age-related deficits

2

in cognitive function are progressive and generalized; however, some studies do show heterogeneous patterns of age-related cognitive decline (Santos *et al.*, 2013). For that reason, the conventional group averaging statistical approach is no longer satisfactory in understanding the true effect of brain aging (Hsieh, 2015). Thus, more than group descriptions and comparisons with younger subjects, it is crucial to consider inter-individual differences and its determinants, often neglected in most of the published studies; in this thesis, as will be shown later, we considered inter-individual differences as central to understand aging.

Throughout the aging process, the desire of a long life goes on pair with that of a healthy one. Considering that the longevity is increasing worldwide and that there is a lack of information about the aging process and its heterogeneity, the understanding of the mechanisms that are responsible for age-related cognitive changes becomes increasingly important, in the hope of finding new therapeutic options for limiting the effects of aging on neuronal function.

We will next overview key aspects related to aging in the brain, namely cognitive function (Chapter I section 2) and relevant brain structures for cognition (Chapter I section 3); we will also highlight in further detail concepts and processes presently known about the aging brain that are most relevant under the scope of this thesis (Chapter I section 4).

2. Cognition and cognitive function

The word "cognition" dates back to the 15th century when it meant "thinking and awareness" (Revlin, 2012). Nowadays, cognition is defined as "those processes by which the sensory input is transformed, reduced, elaborated, stored, recovered, and used" (Neisser, 1967).

In general, human cognition refers to the ability to assimilate and process the information that its received from different sources (perception, experience, belief, etc), and to convert it into knowledge (Revlin, 2012). Likewise, Shettleworth in 1998 argued that "animal's cognition refers to the mechanisms by which they acquire, process, store and act on information from the environment".

Cognition encompasses a variety of cognitive functions, like learning, attention, memory, language, reasoning and decision making (Kandel, 2000). Each of these cognitive functions are responsible for regulation of specific behaviors or actions and are served by more than one neural pathway that has been persistently mapped in the brain (Kandel, 2000). Understanding how these networks produce the cognitive functions of the brain is one of the ultimate challenges of neuroscience. When one functional region, or pathway, is damaged, others may be able to compensate partially for the loss, otherwise, pronounced deficits in specific cognitive functions may emerge. Eventually, this will lead to alterations in

behavior that, altogether, are a reflection of the adjusted capacities of the whole functioning brain, rather than the reflex of the loss of damaged brain regions (Kandel, 2000). Nevertheless, this ability to compensate for lost function depends on the affected region and the extension of the injury. As lesion studies have shown, both in humans and in animal models, severe lesions are often accompanied by specific and reproducible cognitive deficits (Squire & Wixted, 2011).

In this thesis, we will focus specifically on two major domains of cognitive function: learning and memory. While learning is the process by which the brain acquires information from the external environment, memory is the process by which that knowledge is encoded, stored, consolidated and later retrieved (Martin & Morris, 2002). These processes operate conjointly at the level of neural networks (Holtmaat & Caroni, 2016; Kandel *et al.*, 2014).

The study of human memory skyrocketed from the 1950s on with the work performed on the patient H.M. (Squire & Wixted, 2011). As a result, different kinds of memory and learning were distinguished.

Memories can be generally classified according to their content (declarative or nondeclarative), according to their duration (short-term memory or long term-memory), and according to their nature: purely archival (short-term memory or long term-memory) as opposed to working memory, a transient process encompassing both storage and processing functions, the "blackboard of the mind" (Goldman-Rakic, 1996; Squire & Zola, 1996; Rendeiro *et al.*, 2009; Squire & Wixted, 2011) (Figure 1).

Non-declarative (implicit) memory, is rigid, outside of a person's awareness, and tightly connected to the original stimulus conditions (Squire & Wixted, 2011) (Figure 1). This type of memory is related to abilities (procedural memory) such as the ability to retain memory for motor and cognitive skills (Harada *et al.*, 2013). It is dependent on the amygdala, cerebellum, striatum and reflex pathways, and its recall is unconscious (Squire, 1986; Squire & Zola, 1996) (Figure 1). This type of memory is relatively stable across the lifespan (Harada *et al.*, 2013).
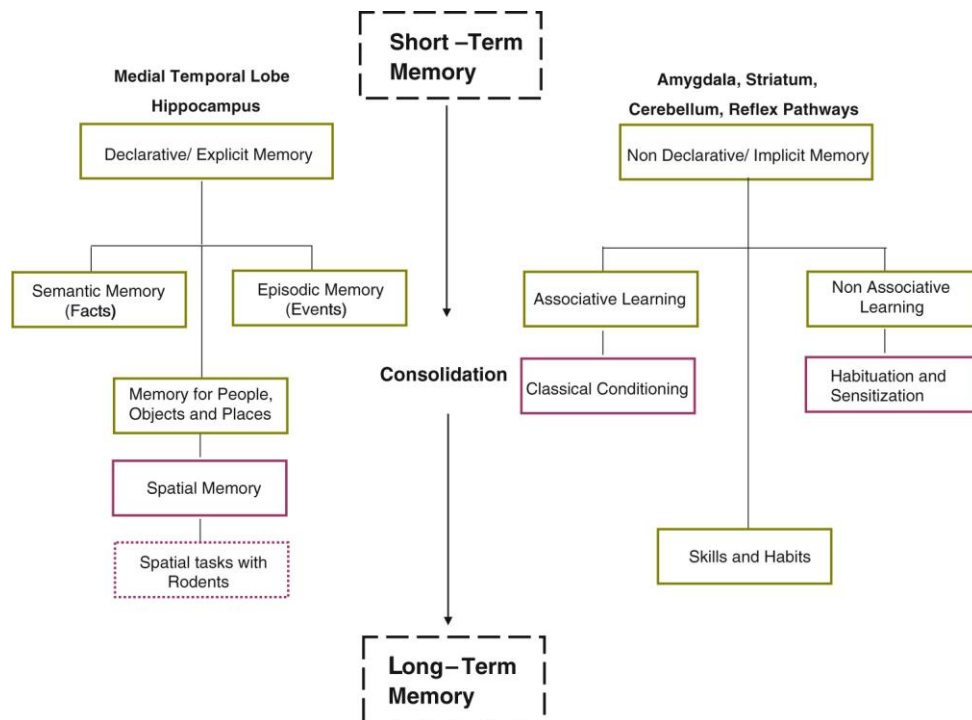
Figure 1| Taxonomy of memory systems. Declarative and non-declarative memories are the two main types of human memory. Declarative memory is further divided in semantic, episodic and spatial memory, while non-declarative memory comprises associative and non-associative learning, skills and habits (procedural memory). Associative learning occurs through the association of two previously unrelated stimuli, and includes reinforcement, whereas non-associative learning occurs in response to a single stimulus, without reinforcement. Short-term memories are consolidated into long-term memories by repetition (Adapted from Rendeiro *et al.*, 2009).

Unlike nondeclarative memory, declarative memory shows a marked impairment throughout the life course (Harada *et al.*, 2013). It is highly flexible and represents the factual knowledge of people, places, objects and events, acting to form associations between them; it is the kind of memory that is referred to when the term memory is used in everyday language (Squire & Wixted, 2011). It is supported by the medial temporal lobe and hippocampus (HPC), and its retrieval requires conscious attention (Squire, 1986; Squire & Zola, 1996) (Figure 1). Examples of declarative memory include semantic memory, episodic memory and spatial memory (Figure 1). Semantic memory involves fund of information, language usage, and practical knowledge, for example, knowing the meaning of words (Glisky, 2007; Harada *et al.*, 2013). Episodic memory refers to the ability of encoding, storing, and consciously recollecting previously learnt events over variable periods ranging from minutes to years (Burgess *et al.*, 2002; Harada *et al.*, 2013). It concerns to our ability to consciously recollect personally experienced

events including the information related to the ongoing external context such as space, time, as well as who was involved (Burgess *et al.*, 2002).

As humans, rodents acquire declarative memories when they 'learn' the required route within a specific maze environment, indicating that they have formed a flexible neural map of the environment to guide their behavior in specific situations (Burgess *et al.*, 2002). This type of declarative memory, termed spatial memory (Figure 1), is present in both humans and rodents and is commonly divided into two main systems: egocentric (subject-centered), relying mainly on the parietal neocortex, and allocentric (object-centered), which is dependent mainly on the integrity of the HPC, and is also commonly termed spatial reference memory (Burgess *et al.*, 2002; Wirt & Hyman, 2017).

The anatomy of memory has been a question of continuous debate within the neuroscience community (Jonides *et al.*, 2008). Particularly, the storage of short-term and long-term memories, as well as the brain regions responsible for the executive processes guiding their integration, are far from being resolved. Nevertheless, the state-based model presently gathers the best consensus. This model assumes that short-term and long term-memories are stored within the same regions of the cortex that where involved in the initial perception and encoding, and that frontal regions are responsible for executive functions (Jonides *et al.*, 2008; LaRocque *et al.*, 2014; Nyberg & Erikson, 2015). In this sense, short-term memories, stored in posterior cortical regions, will be maintained and processed following prefrontal cortex (PFC) recruitment, allowing for updating, mnemonic representations of stimuli, manipulation, and interactions with long-term memories, for example by interactions between short-term and long-term memory storages. Therefore, maintenance of novel information in working memory can engage the medial temporal lobe consolidation system, having the HPC as a central participant, leading to the formation of new long-term memories. The medial temporal lobe is thus a highly specialized region of the brain, able to create representations involving novel relations - encoding, while having a pivotal role in memory consolidation and retrieval. The PFC, in turn, has a pivotal role in working memory, functioning as an online maintainer of information, allowing manipulation, updating, coordination of behavior when multiple goals are active, and shifting from one set of rules to another one, the so called behavior flexibility (de Bruin *et al.*, 1994; Jonides *et al.*, 2008; LaRoque *et al.*, 2014; Nyberg & Eriksson, 2015).

Declines in declarative memory occur with normal aging, however, the timing of these deficits is different. While episodic and spatial memories show lifelong declines, semantic memory shows late life deterioration (Rendeiro *et al.*, 2009; Harada *et al.*, 2013).

Thus, an understanding of the biological basis of these cognitive functions, particularly those under the scope of this thesis, learning and memory, requires an appreciation of the anatomy and function of the

neural systems that subserve these functions in the brain. Therefore, our work was focused in the study of HPC and PFC circuits, since both are critical structures for learning and memory, and both are involved in the process of brain aging. We will next, refer to these brain regions with further detail.

3.   The hippocampus and the prefrontal cortex – implications to cognitive function

During the last few years, the emergence of new imaging techniques revolutionized the study of the neuroanatomical basis of cognitive functions. Thus, a much clearer idea about the brain regions involved in many complex cognitive functions is being formed. In this regard, it is now known that the HPC and the PFC are critical structures for learning and memory (Burgess *et al.,* 2002; Squire & Zola, 1996; Damasio, 2000). Because of their crucial role in memory in general, and in working memory and spatial memory, particularly, these two brain structures have been the major focus of a productive line of research, concentrated in the fundamental aspects of cognitive processing. Here, we present a general survey of the literature, regarding the human and rat brain. For each brain region, we first review the structural and functional organization, and then take a deeper look at the dependent behaviors.

3.1.   Hippocampus – structural and functional organization and dependent behaviors

The HPC is a highly interconnected structure that serves a pivotal role in the formation of declarative memories (Squire, 1992; Morris *et al.*, 2003), as well as in the regulation of the hippocampal-pituitary-adrenal axis (reviewed by Sousa *et al.*, 2008). Due to this fundamental role, atrophy in the HPC formation is often an early feature in the progression of Alzheimer's disease (AD), and patients with damaged HPC often have difficulty in forming new, and enduring memories of personally experienced events. This general episodic amnesia coexists with marked deficits in spatial orientation and navigation (Hartley *et al.*, 2013).

The hippocampal region comprises the HPC formation and the parahippocampal region (Cappaert *et al.*, 2014). In this thesis we will focus on the HPC formation. The HPC formation is a medial temporal lobe structure belonging to the limbic system. It has a long C-shaped form that is present across all mammalian orders and runs along a dorsal (septal)-to-ventral (temporal) axis in rodents, corresponding to a posterior-to-anterior axis in humans (Moser & Moser, 1998; Strange *et al.*, 2014). While the dorsal (or posterior) HPC (DHPC) mediates cognitive functions, particularly spatial memory; the ventral (or anterior) HPC (VHPC) is involved in emotional responses (Moser & Moser, 1998; Fanselow & Dong, 2010). In particular, a more dense ventral than dorsal connectivity with the amygdala (Fanselow & Dong,

2010), and the selective VHPC role in the endocrine stress response (reviewed by Sousa *et al.*, 2008), strongly support this dichotomy in HPC cognitive processing.

The anatomy of the HPC formation comprises three main subfields, distributed from proximal to distal along the transverse axis of the HPC, with the dentate gyrus (DG) as the most medial and proximal portion, laterally flanked by the cornu ammonis (CA), the HPC proper, with its three subfields (CA1, CA2, CA3), and the subiculum (Sb) (Strange *et al*,. 2014). The CA1 and CA3 fields are the HPC regions which were subject to more intense study; in contrast, the smaller CA2 is, so far, the less-studied CA subfield. Indeed, some authors (Lorente de No, 1934) claim that the CA2 subfield consists of CA3-type pyramids which did not receive the mossy fiber contacts coming from the dentate granule cells, while others reported no differences between CA2 and CA3 cells in terms of their connections or histochemical staining properties (Blackstad, 1956). Nevertheless, recent studies have revealed unique properties and an influential role of this region in encoding social, temporal and contextual aspects of memory (Robert *et al.*, 2018).

The aforementioned structures constitute the HPC network, which is primarily a unidirectional network. The HPC receives major inputs from the entorhinal cortex (EC), which projects to the granule cells of the DG and CA3 pyramidal neurons via the perforant path. The axons of the granule cells are termed mossy fibers and send inputs mainly to the apical dendrites of the pyramidal cells of the HPC area CA3. CA3 neurons, in their turn, send axons to the apical dendrites of CA1 pyramidal cells via the Schaffer collateral pathway, as well as to CA1 cells in the contralateral HPC via the associational commissural pathway. CA1 neurons also receive input directly from the perforant path; and they are the last point of this trisynaptic circuit, sending axons to the Sb. Subicular neurons close this circuit by sending backprojections to the EC, forming, therefore, a reverberating loop (Strange *et al.*, 2014; Kesner & Rolls, 2015). This basic intrinsic circuitry is maintained throughout the long axis and across species. Also, a cross-species comparison of anatomical connectivity provides evidence that the primate HPC long axis may be homologous to that of the rat (Strange *et al.*, 2014).

The present thesis will focus on the study of the DG, CA3 and CA1. Although the role of the HPC is most often evaluated as a single entity, recent evidence from a variety of experimental approaches including lesion, behavioral, electrophysiological and gene activation studies, suggests that there are separable and distinct sub-regional functions within this structure (Kesner *et al.*, 2004). It is known that the DG processes metric spatial representation, performing complex pattern separation computations, while the CA3 subregion is responsible for spatial pattern association and completion, detection of novelty and short-term memory. The CA1 subregion mediates processes involved in temporal pattern association and

completion as well as intermediate-term memory, functioning as an important output hub strongly involved in memory consolidation (Kesner *et al.*, 2004).

Furthermore, given the prominent role of the HPC in memory, it is no surprise that the HPC and PFC are anatomically related. Indeed, they are connected through the projection of axons originating in the Sb and ventral CA1 subfields, which terminate in the pyramidal cells and interneurons of the medial PFC (mPFC) (Tierney *et al.*, 2004) (Figure 2). The pathway is unidirectional but may be reciprocated via a bi-synaptic route through the nucleus reunions or lateral EC (Vertes *et al.*, 2007) (Figure 2). Similarly, projections arising from the mPFC are returned to the HPC through a direct connection from mPFC to dorsal CA1 or via the EC through the nucleus reuniens and also via the medial dorsal thalamic nuclei (Wirt & Hyman, 2017) (Figure 2). The integrity of the mPFC-HPC connection is necessary for spatial working memory in paradigms including the water maze (Wang & Cai, 2008), the T-maze (Wang & Cai, 2006) and spatial win-shift on the radial arm maze (Goto & Grace, 2008). Modulation of synaptic activity in the connection between these two areas contributes to a synergistic regulation of learning/memory processes (Cerqueira *et al.*, 2007a).

Summarizing, due to the multiple and disparate functions that have been ascribed to the HPC, it has been intensively investigated in the human and rat brain in the context of understanding memory, learning processes, neurodegenerative diseases and cognitive decline.
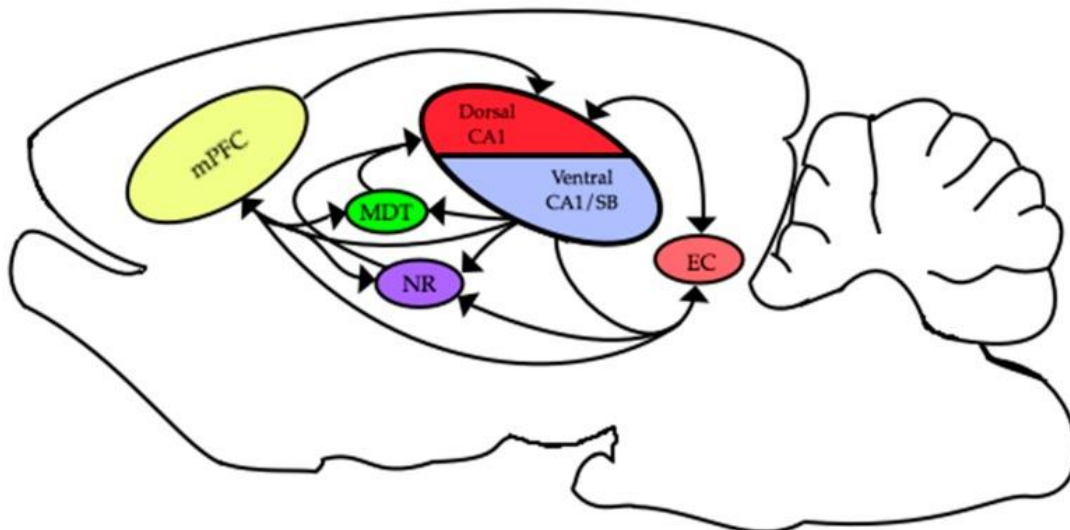
Figure 2| Connections between the HPC and the mPFC. Projecting axons originating in the Sb and ventral CA1 subfields terminate in the pyramidal cells and interneurons of the medial mPFC (for more details see text). medial prefrontal cortex = mPFC; nucleus reuniens = NR; mediodorsal thalamic nuclei = MDT; subiculum = Sb; CA1 = dorsal (top) ventral (bottom). (From Wirt & Hyman, 2017).

3.2.   Prefrontal cortex – structural and functional organization and dependent behaviors

The PFC is critical to many cognitive abilities that are considered particularly human, and is part of a large neuronal system crucial for normal affective behavior and executive functioning, both in humans and other primates (Teffer & Semendeferi, 2012). In general, the PFC plays a key role in decision making (Manes *et al.*, 2002), planning (Muller *et al.*, 2002), processing of emotional stimuli and behavioral flexibility, as well as in social interactions (Damasio, 2000). Also, as already discussed, the PFC has a central role in working memory (Damasio, 2000), which involves transient storage and manipulation of information to guide subsequent behavior. For a long time the PFC was thought unique to the primate species and called the "frontal granular cortex" (Uylings *et al.*, 2003). It comprises a group of cortical areas which are structurally and functionally heterogeneous (Lewis, 2004). It includes all cortical areas of the primate frontal lobe that have an inner granular layer IV and lie rostral to the agranular (pre)motor region. In primates, these areas can be roughly divided into different anatomic subfields namely a dorsolateral, a medial (anterior cingulate) and an orbital region (Uylings *et al.*, 2003; Lewis, 2004). Damage to the human dorsolateral frontal region is characterized especially by deficits in working memory. Damage to the orbitofrontal region is characterized by altered socio-emotional behaviors, hyperkinesis, deficits in the processing of olfactory and gustatory information, and in spontaneity. The medial (anterior cingulate) region is not as well characterized, but its function is related to attentional processes related to internal states. Thus, damage to anterior cingulate regions can include reduced response to pain, akinetic mutism, and impaired motor initiation (Uylings *et al.*, 2003).

While most authors agree on this anatomical distinction, they differ in the different functions that have been ascribed to these subdivisions of the primate PFC, particularly along its dorsal-ventral axis (Goldman-Rakic, 1995; Damasio, 2000). For instance, regarding the contributions of the different PFC subareas to working memory, two principal theories have emerged - the ''type of information'' theory and the ''type of processing'' necessary for task completion (Goldman-Rakic, 1995; Muller *et al.*, 2002). In the first theory, the primate dorsolateral PFC is ascribed to spatial memory content whereas more ventral areas subserve memory for objects. On the other hand, in the "type of processing" necessary for task completion theory, PFC ventrolateral regions would subserve active maintenance of both object and spatial information within memory, whereas the dorsolateral region is required for ''monitoring'' and ''manipulation'' of working memory information (Muller *et al.*, 2002).

The volume of the cerebral cortex of a rat is about a hundred times smaller than that of the cerebral cortex of macaques, and about a thousand times smaller than that of humans. This decreased volume is paralleled by less evolution, less differentiation, and less segregation than the corresponding primate

10

cerebral cortex. This fact has raised controversy whether or not rats possess a prefrontal region comparable with the primate PFC. Nevertheless, the use of other classification aspects rather than the sole use of cytoarchitectonic criteria has progressively put aside these early disagreements about the existence of a PFC in non-primates. Thus, it is now generally accepted that connectivity, functionality, neurotransmitter milieu and embryological development should be considered, together with cytoarchitectonic characteristics, when discussing homologies between cortical areas in different species (Uylings *et al.*, 2003). Taking these criteria under consideration, one can delineate a region on the frontal pole of the rat brain that may be considered equivalent to the primate PFC (Uylings *et al.*, 2003).

The rodent PFC can be grossly divided into two main regions: a medial region (mPFC), that has characteristics of both dorsolateral and medial primate subdivisions, and a lateral and ventral subfield, equivalent to the primate orbital region (OFC) (Heidbreder & Groenewegen, 2003; Dalley *et al.*, 2004; Zilles & Wree, 2004). The rat mPFC, as a whole, has been traditionally implicated in attentional processes, working memory and behavioral flexibility, therefore, throughout the rest of this thesis, together with the HPC, this brain region will be the focus of our study.

The rodent mPFC receives diverse afferent inputs from limbic regions, including the amygdala and HPC (CA1 and Sb), and provides direct outputs to hypothalamic and numerous brainstem areas involved in the regulation of emotion and the physiological response to stress (Bandler *et al.*, 2000). It can be further divided into the frontal area 2, dorsal and ventral anterior cingulate areas (Cg), prelimbic area (PL), infralimbic area (IL) and medial orbital area (MO) (Van Eden, 1985). Furthermore, there is now consensual agreement on a main subdivision of the mPFC into dorsal (mainly Cg and PL) and ventral (mainly IL) components. While ventral regions are specialized for autonomic/emotional control, dorsal regions regulate working memory and some forms of motor sequencing (Uylings *et al.*, 2003; Heidbreder & Groenewegen, 2003).

Thus, the anterior Cg cortex and the dorsal PL area have been implicated in attention and working memory (Cerqueira *et al.*, 2008; Euston *et al.*, 2012; Heidbreder & Groenewegen, 2003); while the ventral IL area has been associated to goal-directed behavior and autonomic functions (Heidbreder & Groenewegen, 2003; Euston *et al.*, 2012). Both PL and IL areas have been associated with the performance in behavioral flexibility tasks (Ragozzino *et al.*, 1999).

In summary, the rat PFC is subdivided into a ventrolateral area (OFC area), that plays a central role in the control of socio-affective behaviors; a dorsomedial area (mainly Cg and PL) that regulates working memory, and a ventromedial (mainly IL area) that is involved in visceromotor behaviors. The present

thesis will focus on the analyses of the two latter subdivisions, which represent the main bulk of the rodent mPFC.

4. The aging brain

"The aging process is a biological reality which has its own dynamics, largely beyond human control" (Gorman, 1999). This statement is only one of the many that can be used to define the process of aging. Indeed, for many years, scientists have been looking for a single theory that explained the aging process. However, no single unifying theory may exist, since the mechanisms of aging can be quite distinct in different organisms, tissues and cells; rather, there are currently over 300 theories to explain the aging phenomenon (Tosato *et al.*, 2007; Jin, 2010). This diversity reveals a profound truth about its nature: aging is a complex interaction of genetics, biochemistry, physiology and behavior, which heightens the difficulty of finding a universal theory. Mostly, these aging theories could be divided into two main groups: the first states that aging is natural and programmed into the body, while the second says that aging is a result of the burden exerted on the body, through cumulative attempts to adapt to life demands – the allostatic load, such as the impact of cumulative stress (McEwen & Seeman, 1999).

In general, aging is associated with the gradual deterioration of biological systems including the brain. Since the aging brain is the major risk factor for most common neurodegenerative diseases (Yankner *et al.*, 2008), including AD, cerebrovascular disease, Parkinson's disease (PD) and amyotrophic lateral sclerosis, the study of normal brain aging is extremely important as an effort to provide a backdrop against which pathological neural changes can be interpreted. Also, since the number of elderly people is continuously increasing, it will become increasingly important to understand the cognitive changes that accompany aging, both in normal and pathological scenarios.

It is known that the anatomical and functional organization of the human brain is dynamic and changes in response to many stimuli. One of them is the natural process of aging, which inevitably triggers plastic and adaptive changes in the brain (Dickstein *et al.*, 2007). While some alterations in the architecture of the brain do not necessarily signal a pathological change, on the other hand, when the brain is not able to compensate for accumulation of insults, a decline in cognitive abilities may occur (McEwen & Seeman, 1999; Dickstein *et al.*, 2007). Indeed, although the overall picture might seem to be one of cognitive decline, some people can retain excellent memory skills into advanced old age. Despite of the knowledge about the neurobiology of learning and memory processes, there is no consensus on the precise nature of age-related cognitive decline. For instance, some authors believe that the decline in cognitive function can, in part, be explained by changes in neural plasticity or cellular alterations that directly affect

mechanisms of plasticity (Erickson & Barnes, 2003; Burke & Barnes, 2006), while other studies also highlight the role of glucocorticoids in cognitive aging – the "glucocorticoid cascade" theory of aging (Lupien *et al.*, 1994; Nichols *et al.*, 2001; Seeman *et al.*, 2001). Although these mechanisms probably represent only part of the aging process, their study may be the harbinger of important developments in the understanding of age-related changes in cognition.

The increase in lifespan, along with the wide and non-uniform spectrum of cognitive capacity in the elderly, has prompted general questions of great interest to aging researchers about how the brain ages, which structures change, at what rates, when do they start aging, and how does its integrity deviates. These are some questions of utmost importance that need to be clarified.

In this thesis, we have focused our interest on determining how brain structures crucial for cognition, such as the HPC and the mPFC, change with age, and how factors such as cognitive training impact these changes (Milgram *et al.*, 2006). Since the study of the human brain is difficult, for many reasons including ethical issues, performance of invasive studies, controlling for all genetic and environmental variables among individuals, in this project, we will study the aging brain by using rats as models of cognitive aging.

4.1.   Normal brain aging versus pathological brain aging

Aging is most conveniently defined as a multidimensional process of physical, psychological, and social changes during an individual's lifespan that leads to the declining ability to respond to stress, increased homeostatic imbalance and increased risk of disease eventually ending in death (Tosato *et al.*, 2007). These inevitable natural changes that aged individuals experience in their diverse biological systems, including the brain, are not necessarily harmful and this process is called - Normal aging. On the other hand, when the adaptive responses to challenge lie chronically outside the normal operating ranges, wear and tear on regulatory systems occurs and allostatic load accumulates, ultimately leading to disease - Pathological aging (like AD and PD) (Seeman *et al.*, 2001;Fjell *et al.*, 2014).

While research in the area of normal cognitive aging may seem less pressing than research in the area of pathologic brain disease, a more complete understanding of normal brain aging may shed light on important abnormal brain processes.

The distinction between normal and pathological brain aging in humans tends to be difficult because several of the age-related neurobiological changes identified during the normal aging process are subtle compared with the alterations observed in age-associated disorders (Fjell *et al.*, 2014). Studies performed in animals have helped to disambiguate the boundary between normal and pathological states of aging

in humans (reviewed by Erickson & Barnes, 2003). These normal cognitive changes are essential to understand because, on one hand, they can affect an older adult´s day to day function, and on the other hand, they can help us distinguish normal from disease states. This differentiation is important to identify pathological states, and to allow for therapeutic measures to be taken when appropriate.

Additionally, since normal aging is a complex issue, we must also be able to identify the true effects of aging and how to separate factors such as socioeconomic disadvantage, lack of educational opportunity and cognitive engagement (Paulo *et al.*, 2001; Stine-Morrow *et al.*, 2008; Josefsson *et al.*, 2012; Santos *et al.*, 2013).

In the present study, rats will be used to model fundamental aspects of human memory, and to address age-related cognitive changes occurring during the normal aging process. Moreover, understanding age-related changes in cognition sets a background against which it is possible to assess the effects of pathological disease states.

4.2. Neurocognitive changes in aging

As individuals get older, several changes occur in the structure and function of the brain. The inability to compensate for the accumulation of insults occurring throughout the lifespan, ultimately leads to alterations in cognitive performance. Since cognitive ability is a crucial determinant of the quality of life of elderly people, the study of these age-related cognitive deficits sharply increased.

Although as discussed above, some aspects of cognition remain relatively intact with age, there is now consensus that certain aspects of learning and memory decline with progressing age. The HPC and mPFC are critical structures for learning and memory (Damasio, 2000; Squire & Zola 1996), and are particularly vulnerable to the aging process. So, it is expected that the performance in tasks that require information processing in these brain regions declines in the course of normal aging.

Indeed, in humans, the cognitive domains most affected by normal aging, and the ones most susceptible to brain damage, are associated with executive control, and episodic and spatial memory; other cognitive processes are relatively unaffected, namely semantic memory and verbal abilities (Raz, 2000; Glisky, 2007; Nyberg *et al.*, 2012; Fjell *et al.*, 2014). However, there is disagreement about whether longitudinal changes in older adults reflect continuous ongoing processes starting in young adulthood, or whether changes begin in middle age or even beyond (Fjell *et al.*, 2014).

According to Bäckamn (2008), in humans, episodic memory loss is typically the first sign that something may be amiss. In his work, he proposed that relatively poor episodic memory may be an early marker for mild cognitive impairment; but equally, older adults with poor memory abilities may be at risk to develop

dementia (Bäckamn, 2008). Another study reports that episodic memory declines from about the age of 50-60 years on a population basis (Nyberg *et al.*, 2012).

Animal studies have, as limitation, the fact that animals cannot describe their experiences directly, making the investigation of certain cognitive functions, such as episodic memory, difficult. To make inferences about the aging process from animal models back to humans, rigorous behavioral paradigms must be used to ensure that the same function is being examined across species. Fortunately, the cognitive domain of spatial memory, which changes during normal aging, provides a common ground between species, and is a domain where age-related deficits are described consistently for humans (Uttl & Graf, 1993), nonhuman primates (Rapp *et al.*, 1997), and other species such as dogs (Head *et al.*, 1995), mice (Gallagher & Rapp, 1997) and rats (Ménard & Quirion, 2012). Thus, it is tempting to suggest that the mechanistic understanding of the neural alterations described in animal models may, one day, lead to a deeper understanding of brain aging in humans.

Nowadays, rats have been extensively used as models of cognitive aging (Syková *et al.*, 2002; Ménard & Quirion, 2012; McQuail & Nicolle, 2015). This can be partially explained by their ability to serve as a good model to test spatial memory, due to their impressive skills of orientation in the surrounding environment, while remembering complex relationships between visuospatial cues, in a way very similar to humans (Rendeiro *et al.*, 2009). Indeed, several studies reported that aged male rats (22–24 months-old) show impairments, as compared to young rats (2–4 month-old young), in a number of spatial memory tasks including: Y and T mazes (Aggleton *et al.*, 1989), radial arm maze (Luine & Hearns, 1990) Morris water maze (MWM) (Gallagher *et al.*, 1993, Ménard & Quirion, 2012; McQuail & Nicolle, 2015), water radial arm maze (Bimonte *et al.*, 2003) and Barnes maze (Barret *et al.*, 2009).

In this thesis, we used the MWM task described in 1984 by Richard Morris to assess spatial learning and memory in rodents, as well as some variations of this task, designed to assess working memory and behavioral flexibility. A similar task was described for humans, providing a solid basis for cross-species comparisons (Hamilton *et al.*, 2002).

In summary, preserved cognitive performance is assumed as a vital feature of successful aging. Thus, the study of the relationship between brain integrity and memory in healthy elders is of paramount importance to understand the evolution of cognitive function during the aging process. So far, the substrates of age-related cognitive deficits remain uncertain. Taking this into account, the structural, functional and molecular alterations occurring during the aging process will be explored next.

4.3. Heterogeneity in cognitive function during the aging process

The aging process occurs in everyone; however, no one ages in the same way or at the same rate. Hence, one of the most striking characteristics of cognitive aging is its heterogeneity (Ardila *et al.*, 2003). In general, normal aging is associated with cognitive decline, and even excluding pathological aging (dementia and other illnesses associated with age), an enormous variability exists across individuals. Some individuals maintain an adequate function until later in life and perform as well or better than younger adults, while others, of the same age, present signs of cognitive decline (Ardila *et al.*, 2003; Josefsson *et al.*, 2012; Harada *et al.*, 2013; Santos *et al.*, 2013) (Figure 3).
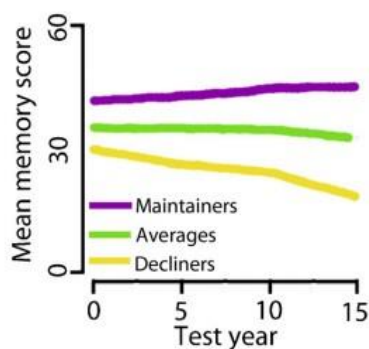
Figure 3| Longitudinal episodic memory decline during aging. Notice the heterogeneity observed in the aging process. Elderly people were divided into three groups in terms of memory change - maintainers (people that maintain their functional level); those who showed age-typical decline and decliners (Adapted from Fjell *et al.*, 2014).

Additionally, decline is not uniform across cognitive domains, with some cognitive processes being affected by normal aging, while others remain unaffected. For example, some older adults have excellent episodic memory function but impaired executive function, and vice versa (Glisky *et al.*, 1995). Thus, although there are clear interactions among cognitive domains, it seems evident that they also have some degree of independence, being differently affected in different individuals. In the recent years, a question that has been puzzling aging researchers is what accounts for this variability.

This inter/intra-individual variability may arise from age-related structural and physiological changes in the brain (Raz, 2000). In addition, the interaction of multiple factors and mechanisms, such as biological, psychological, health-related, environmental, and lifestyle, may have implications in the heterogeneity of cognitive function (the last two factors will be further addressed in detail in section 4.6) (Paulo *et al.*, 2001; Stine-Morrow *et al.*, 2008; Josefsson *et al.*, 2012; Santos *et al.*, 2013). In a very interesting study, longitudinal trajectories in episodic memory were mapped over 15 years in more than 1500 participants

16

from the Betula study (Josefsson *et al.*, 2012). In this work, it was shown that some individuals age more successfully than others, and the researchers were able to identify environmental and genetic factors predicting group membership. Thus, when neuropsychological tests are performed to assess cognitive function, it's important to be aware of all the variables capable of promoting alterations in cognitive function.

The current use of rats as models of cognitive aging allows a more restricted control of such variables. Surprisingly, most studies in animals failed to accommodate this evidence of individual heterogeneity in aging and, apart from a few examples, have considered old animals as a homogeneous group.

In summary, since cognitive ability is a crucial determinant of elderly people life quality, a thorough understanding of the mechanisms underlying its heterogeneity, during the normal aging process, is of paramount importance to promote healthy aging. Indeed, understanding why some individuals' cognition seems to be unaffected by age could promote the design of strategies to prevent cognitive abilities from declining throughout our lifespans.

4.4. Structural alterations in the aging brain

The well-documented age-related behavioral deficits are concomitant, and seem to be correlated with significant structural alterations in the basic elements of the central nervous system (CNS) (Raz, 2000). A common misconception about normal aging is that significant loss of neurons in the HPC and mPFC areas (Brody, 1955; Rapp & Gallagher, 1996; Rasmussen *et al.*, 1996) occurs. Indeed, evidence from more recent studies, due to the use of ''design-based'' stereology procedures, has contradicted these initial findings (Uylings & de Brabander, 2002). Instead of neuronal loss, it is widely accepted that aging is accompanied by an overall brain volume loss, in both humans (Driscoll *et al.*, 2003, 2009; Freeman *et al.*, 2008; Raz *et al.*, 2010) and rats (Rapp *et al.*, 1999; Driscoll *et al.*, 2006). Moreover, several studies reported age-related cognitive decline to be associated with volume loss, dendritic atrophy, and changes in spine density in areas implicated in cognitive abilities, such as the HPC and the mPFC (Driscoll *et al.*, 2003, 2006; de Brabander *et al.*, 1998; Markham & Juraska, 2002; Dickstein *et al.*, 2013).

4.4.1. Volumetric alterations

Healthy brain aging, even in reasonably performing adults who showed no gross cognitive decline, is characterized by an overall atrophy associated with decrease in gray and white matter volumes and expansion of ventricular spaces (Raz *et al.*, 2005; Tammes *et al.*, 2013). In fact, several studies have

already shown that volumetric alterations in areas implicated in cognitive abilities could be an important determinant for elderly cognitive function (Driscoll *et al.*, 2003, 2006; reviewed by Dickstein *et al.*, 2007). It is also known that specific brain regions are more susceptible to shrinkage than others, and that changes in brain volume are not uniform across areas/individuals (Raz *et al.*, 2005). In this regard, the HPC, frontal areas and striatum, are among the regions most affected by age in healthy humans (Raz *et al.*, 2005; Peters, 2006). Historically, it has been presumed that brain volume loss in HPC and mPFC areas underpin age-related cognitive decline, in both humans (Jernigan *et al.*, 1991; Golomb *et al.*, 1993 Driscoll *et al.*, 2003; 2009; Freeman *et al.*, 2008; Raz *et al.*, 2010) and rats (Rapp *et al.*, 1999; Driscoll *et al.*, 2006; Yates *et al.*, 2008). While HPC atrophy has been associated with deficits in episodic and spatial memory (Golomb *et al.*, 1993; Van Petten, 2004), PFC atrophy is associated with deficits in executive function and working memory (Cerqueira *et al.*,2005). The implicit link for aging is, of course, an existence of volumetric atrophy which impairs function. However, despite the several studies already addressing volume loss and cognitive deficits during the aging process, contradictory findings constantly challenge this common assumption (Sullivan *et al.*, 1995; Raz, 1996; Van Petten, 2004), which could be justified by multiple factors that affect the course of brain aging. For instance, Raz *et al.* (2003) showed that hypertension exacerbates age-related shrinkage in prefrontal regions; Coffey *et al.* (1998) reported that women show lesser brain aging than men; Satz (1993) demonstrated that larger brain volume is a neuroprotective factor, and other studies reported that higher formal education delays brain aging and ameliorates its course (Stern *et al.*, 1992; Kramer *et al.*, 2004). Likewise, it is important to highlight that the heterogeneity observed in the aging brain has not been a focus of attention in previous aging studies, which could possibly represent a significant confounding factor.

Given the above, the experimental work presented in this thesis (Chapter III) was carried bearing in mind the possible relation between normal aging heterogeneity and the consequent brain atrophy and cognitive decline, with a bias towards the mPFC and HPC, as these brain regions were shown to be the most commonly affected during the aging process. Moreover, as there is evidence of an existing relationship between neuronal remodeling and volumetric alterations (Cerqueira *et al.*, 2005, 2007b), we also tackled the relation between neuronal dendritic branching and cognitive performance.

4.4.2.   Dendritic alterations

Neuronal dendritic trees are important in the formation and maintenance of neural networks, regulation of synaptic plasticity and the integration of electrical inputs (Jan & Jan, 2010). Therefore, alterations on their structure impact on cognitive function. More importantly, changes in their morphology were shown

to correlate with cognitive alterations in both rodents and humans (see as an example Becker *et al.*, 1986 and Cerqueira *et al.*, 2005).

In this regard, a large body of literature has examined the state of neurons during aging and has shown contradictory results. Some studies reported an age-related regression in the dendritic arbors of the neuronal populations in the HPC and mPFC, while others showed the opposite or even an absence of dendritic remodelling (see table 1 and 2 for more details). Therefore, similarly to the conclusions redrawn from volumetric studies, several studies have also reported age-related cognitive decline to be associated with dendritic atrophy in areas implicated in cognitive abilities, such as the HPC and the mPFC (Driscoll *et al.*, 2003, 2006; de Brabander *et al.*, 1998; Markham & Juraska, 2002; Dickstein, 2013). However, as before, apparent contradictions between studies undermine the establishment of a general consensus.

Table 1: Morphological alterations in HPC neurons during the aging process.

Brain region	Neuron type	Specie/Strain	Gender	Age/Number of individuals/ Number of neurons	Dendritic alterations	Method of analysis	Reference
HPC (DG) (CA2-CA3)	Granular	Humans (Normal aged)	Male/ Female	Middle-age (43-60y), n=7; old adults (68-79y), n=5; very old adults (86-95y), n=5. 15 neurons per case.	Dendritic extent was found to increase between middle age (fifties) and early old age (seventies). However, apical dendritic regression (more than 40%) was found in the oldest old (nineties). No change in dendritic morphology.	Golgi-Cox staining	Flood *et al.*, 1985, 1987
HPC (DG,CA1, CA3) Entorhinal cortex	Granular Pyramidal	Humans (Normal aged and senile deterioration)		69-102y, n=9.	Regression of apical dendrites in DG area and both apical and basal dendrites in CA1 and CA3 area. Regression of both apical and basal dendrites.	Golgi-Cox staining	Scheibel *et al.*, 1976, 1979
HPC (CA1)	Pyramidal	Humans (Normal aged and Alzheimer´s disease)	Male/ Female	Normal aged (43-95y), n=17; Alzheimer´s disease (70-81y), n=5. 15 neurons per case.	No change in dendritic morphology.	Golgi-Cox staining	Hanks & Flood, 1991
HPC (CA1)	Pyramidal	Humans (Normal aged)	Male/ Female	48-74y, n=8 (M); 53-68, n=5 (F).	No changes in the total dendritic length, number of dendritic segments and average segment length.	Golgi-Cox staining	Barrera *et al.*, 2001
Parahippo-campal Gyrus	Pyramidal (layer II)	Humans (Adult, normal aged, senile dementia)		51.2y, n=5; 79.6, n=5; 76.0, n=5. 15 neurons per case.	Normal aged individuals had longer and more branched dendrites than either adult or senile dementia individuals.	Golgi-Cox staining	Buell & Coleman, 1979, 1981
HPC (DG)	Granular	Rat (Fischer-344)	Male	3mo, n=5; 25mo, n=5.	In aged animals, the number, volume fraction and surface area of dendritic profiles are significantly decreased.	Electron microscopy	Geinisman *et al.*, 1978
HPC (DG)	Granular	Rat (Fischer-344)		3mo; 12-20mo; 27-20mo.	No significant change in dendritic length of neurons between groups, with a trend towards an increase between middle-age (20mo) and old age group (27mo).		Flood, 1993
HPC (CA1)		Rat		3mo; 24mo.	In aged rats, the number of dendritic branches decreased by 38%, the total volume fraction and total surface of dendrites per volume neuropil were decreased by 23% and 35%. The percentage area of neuropil occupied by dendritic branch profiles was smaller in aged rats (by 22%).	Quantitative ultrastructural methods	Lolova et al. 1989
HPC (CA1)	Pyramidal	Rat (Fischer-344)	Female	3mo, n=41; >26mo, n=12.	No change in dendritic morphology.	Horseradish peroxidase	Turner & Deupree, 1991
HPC (CA1)	Pyramidal	Rat (Fischer-344)		2mo, n=20; neurons n=11 24mo, n=53; neurons n=15	In aged rats an increase in dendritic length was observed, both for basal dendrites and entire neurons, together with an increase in the complexity of CA1 pyramidal neurons.	Intracellular injection of Neurobiotin	Pyapali & Turner, 1996
HPC (CA1)	Pyramidal	Rat (Long-Evans)	Male Female	3-5mo, n=6; 19-22mo, n=5. 3-5mo, n=10; 19-22mo, n=5. 8-15 neurons per case.	In aged rats, a regression of apical dendrites and a decrease in complexity were reported. No change in neuronal complexity was observed.	Golgi-Cox sating	Markham *et al.*, 2005
HPC (CA1)	Pyramidal	Rat	Male	2-3mo, n=25; 18-20mo, n=15. 8 neurons per region.	Decrease in total, apical and basal dendritic length; reduction in the number of terminal endings.	Intracellular injection of Lucifer Yellow	Chen *et al.*, 2014
HPC (DG) (CA1,CA3)	Granular Pyramidal	Mouse (C67B1/6J)	Male/ Female	1-25mo, n>200.	A loss of dendritic arborization was observed with age. In aged animals, a regression of the basilar dendritic apparatus and a decrease in the number of apical branches was observed.	Golgi-Cox sating	Machado-Salas & Scheibel, 1979
HPC (DG)	Granular	Monkey (Rhesus)	Male/ Female	<11y, n=11; >24y, n=9. 40 cells from young and 16 cells from aged monkeys.	Reduced vertical dendritic extents and distal dendritic branching and an increased proximal dendritic branching was observed in the aged group.	Intracellular injection of Biocytin	Luebke & Rosene, 2003

Table 2: Morphological alterations in mPFC neurons during the aging process.

Brain region	Neuron type	Specie/Strain	Gender	Age/Number of individuals/Number of neurons	Dendritic alterations	Method of analysis	Reference
PFC	Pyramidal (layers III and V)	Humans (Normal aged)		58-96y, n=10.	Normal aging leads to loss of both basal and apical dendritic trees.	Golgi-Cox staining	Scheibel *et al.*, 1975
PFC (primary motor cortex)	Pyramidal cells of Betz (layer V)	Humans (Normal aged)		74-102y, n=7	Aging leads to a decrease in the total number of dendritic shafts and the shortening of those which remain.	Golgi-Cox staining	Scheibel *et al.*, 1977
PFC (area 4, motor cortex)	Pyramidal (layers III and V)	Humans (Normal aged)		14-49y, n=5; 52-69y, n=10; 70-79y, n=6; 80-96y, n=7. 30 neurons per region.	Decreased number of basal dendrites. The decrease was more prominent on layer V (about 37%, while in layer III only 13%).	Golgi-Cox staining	Nakamura *et al.*, 1985
PFC (area 10) Occipital cortex (area 18)	Pyramidal (layer I, II, III, IV)	Humans (Normal aged)	Male/Female	14-106y, n=26 (13 male and 13 female). 10 neurons per region.	Regression of dendritic trees of about 10%; decrease in complexity.	Golgi-Cox staining	Jacobs *et al.*, 1997
PFC (area 9 and 46)	Pyramidal (layer III and V)	Humans (Normal aged)	Male/Female	49-90y, n=8. 15 neurons per region.	Regression of layer V dendritic length and decreased complexity. No change in the neurons of layer III.	Golgi-Cox staining	De Brabander *et al.*, 1998
PFC (area 10)	Pyramidal (layer I, II, III, IV)	Humans (Normal aged)	Male/Female	11-69y, n=10 (5 male and 5 female). 10 neurons per region.	No change in dendritic length.	Golgi-Cox staining	Jacobs *et al.*, 2001
mPFC (Cingulate and prelimbic area)	Pyramidal (layer II/III or V)	Rat (Brown Norway)	Male	2mo, neurons n=7-25; 8mo, neurons n=9-24; 18mo, neurons n=20-34; 28mo, neurons n=17-28.	Superficial, but not deep, pyramidal neurons exhibited ongoing dendritic growth after 2mo of age and then dendritic regression after 18mo of age.	Mini-ruby staining	Grill & Riddle, 2002
mPFC (Anterior cingulate area)	Pyramidal (layer V)	Rat (Long-Evans)	Male / Female	3-5mo, n=8; 20-24mo, n=5. 3-5mo, n=15; 20-24mo, n=15. 15 neurons per region.	Reduction of both apical and basal dendrites; decrease in complexity.	Golgi-Cox staining	Markham & Juraska, 2002
mPFC Parietal cortex (LtPA)	Pyramidal (layer III and V)	Rat (Fischer-344)	Male	6mo, n=12; 24mo, n=93. 10-20 neurons per section, 4 sections.	Atrophy of distal basal dendrites (layer V of the LtPA and layer III of mPFC) that did not differ between aged cognitively unimpaired and aged cognitively impaired animals.	Intracellular injection of Lucifer Yellow	Allard *et al.*, 2012
PFC	Pyramidal (layer III and V)	Rat	Male	2-3mo, n=25; 18-20mo, n=15. 8 neurons per region.	Decrease in total, apical and basal dendritic length on neurons from layer IIII and V; reduction in the number of terminal endings.	Intracellular injection of Lucifer Yellow	Chen *et al.*, 2014
PFC (Prelimbic)	Pyramidal (layer II and III)	Rat (Sprague Dawley)	Male	4mo, 21mo	No change in dendritic morphology.	Intracellular injection of Lucifer Yellow	Anderson *et al.*, 2014
PFC	Pyramidal (layer V)	Rat (Long-Evans)	Male/Female	11mo, n=37; 11mo, n=32. 8 neurons per case.	Middle-aged animals had more extensive basilar trees and more branch points than aged animals.	Golgi-Cox staining	Kougias *et al.*, 2016
mPFC	Pyramidal (layer II)	Mouse (SAMP10 and SAMR1)	Male	3mo, n=10; 8mo, n=8; 12-13mo, n=6, 16-17mo, n=8; 20-21mo, n=2. 10-16 neurons per region.	45% decrease in apical length; preservation of complexity.	Golgi-Cox staining	Shimada *et al.*, 2006
PFC	Pyramidal (layer III and IV)	Monkey (Rhesus)		7-28y, n=9. 60 neurons from the 9 animals.	Loss of whole dendritic branches on the apical portion and distoproximal-type degeneration process in basal dendrites.	Golgi-Cox staining	Cupp & Uemura, 1980
PFC (area46)	Pyramidal (layer III and V)	Monkey (Erythrocebus patas)	Male / Female	10-12y, n=1; 21-25y, n=2. 10-12y, n=2; 21-25y, n=1.	No change in dendritic morphology.	Intracellular injection of Lucifer Yellow	Page *et al.*, 2002
PFC (area 46)	Pyramidal (layer III)	Monkey (Macaque) (Rhesus)	Male/Female Male/Female	10-12y, n=3; 10-12y, n=2. 12y, n=1; 24-25y, n=2; 24-25y, n=3. 6 neurons per region.	Reduction of apical dendrites; decrease in complexity.	Intracellular injection of Lucifer Yellow	Duan *et al.*, 2003; Kabaso *et al.*, 2009
Lateral PFC (area 46)	Pyramidal (layer III)	Monkey (Rhesus)	Male Female	8y, n=3; 22.4y, n=1 8y, n=1; 22.4y, n=1	Smaller total apical dendritic length and mean length of unbranched segments in the apical trees.	Fluorescence microscopy	Coskren *et al.*, 2015
PFC	Pyramidal	Dog	Male	2-4y, n=3; 13-18y, n=6.	Regression of dendritic trees.	Golgi-Cox staining	Mervis, 1978
		Caenorhabditis elegans			Neurons in aging animals frequently displayed ectopic branches with increased prevalence with time.	Fluorescent reporters	Tank *et al.*, 2011; Toth *et al.*, 2012

Nevertheless, taken together, these age-related morphological changes seem to be selective, although without a universal pattern across the entire brain. This general lack of agreement in findings could be ascribed to the critical forgetfulness of the heterogeneity observed in the aging brain.

In sum, several studies reported that atrophy of dendritic branching and volume loss in the HPC and mPFC underpin age-related cognitive decline (de Brabander *et al.*, 1998; Markham & Juraska, 2002; Driscoll *et al.*, 2003, 2006; reviewed by Disckstein, 2013). However, despite these reports of age-related structural variations, little is known regarding the relationship between these variations and age-related decline in HPC and mPFC-dependent learning and memory. Also, even acknowledging the evidence for an age-associated decline of cognitive functioning, on average, one cannot oversee the fact that some aged individuals, both humans and rodents (Gallagher *et al.*, 2003; Ardila, 2007; Ménard & Quirion, 2012; Santos *et al.*, 2013), seem to perform as well as their young counterparts. Therefore, in the present thesis, we decided to tackle this question and explore cognitive aging and its structural correlates from an individual perspective in a very large group of aged and young male Wistar Han rats. By addressing inter-individual variation within each age group we expected to tackle its underpinnings and identify some of its determinants.

4.5.   Autophagy, dendritic pruning and the aging brain

Given the described above (section 4.4) on the dendritic alterations that occur during aging, it is worth of mention that in the developing brain, the formation of dendritic trees is followed by selective pruning (Puram *et al.*, 2011). Hence, the homeostasis of the mammalian neuroarchitecture is a dynamic process involving a balance between synaptic sprouting and pruning. The mechanisms underlying these processes are particularly active during development and pathological neurodegeneration but are also functional in physiological conditions (Wong & Ghosh, 2002; Querfurth & LaFerla, 2010;Tang *et al.*, 2014); one such example is the activity-dependent remodeling of synapses and dendritic trees, vital for multiple brain functions including learning and memory (Wong & Ghosh, 2002; Yin & Yuan, 2015; Sugie *et al.*, 2018). Of note, under normal circumstances, the relative importance between dendritic sprouting and pruning varies throughout the lifespan, with synapse and dendritic formation generally exceeding pruning during brain development, while an opposite trend is observed in the adult brain (Puram *et al.*, 2011;Tang *et al.*, 2014). Curiously, a review from Van Petten, 2004, interestingly discusses that the loss of cortical gray matter during childhood and adolescence is responsible for an improvement in cognitive abilities. On the other hand, an excessive dendritic arbor is thought to be associated with neurodevelopmental disorders such as autism spectrum disorders (ASD) (Tang *et al.*, 2014; Kim *et al.*,

2017). Taken together, these evidences highlight the fact that, as for the previously described contradictions on age-dependent morphological remodeling, the significance of the balance between sprouting and pruning is still largely unknown.

The maintenance of sprouting and pruning balance is under tight control through protein synthesis and autophagic recycling (Bingol & Shang, 2011; Puram *et al.*, 2011; Tang *et al.*, 2014; Kanamori *et al.*, 2015). Autophagy, a lysosome-dependent degradation mechanism, is thus as an important catabolic process that, by degrading long-lived synaptic proteins and damaged organelles, importantly impacts on dendritic turnover and synaptic function (Rubinsztein *et al.*, 2011; Shehata & Inokuchi, 2014; Gupta *et al.*, 2016). Therefore, synaptic pruning has a critical role in maintaining CNS homeostasis through the modulation of neuronal density, synaptic remodeling and structural plasticity. Thus, in parallel to the study of classical strategies to prevent cognitive decline, such as cognitive training, which we will explore in the following section (section 4.6) of this introduction, our work was also directed towards the study of alternative approaches such as the autophagy system. Our results (Chapter II) undoubtedly support that the modulation of the autophagy molecular avenue is a promising target for future age therapy interventions.

4.6. The role of cognitive training on cognitive function

Since memory impairments impose a substantial burden for the affected individuals, the development of interventions to maintain and optimize cognitive functioning is of utmost importance.

Several studies have tried to tackle on strategies to prevent cognitive decline in old age, suggesting interventions to achieve successful cognitive aging based on: i) nutritional improvements (Richards *et al.*, 2002), ii) physical exercise (Tanigawa *et al.*, 2014), iii) participation in certain activities - the lifestyle-cognition hypothesis (Marioni *et al.*, 2012), building cognitive reserve (Stern, 2002) and cognitive training (Willis *et al.*, 2006; Nouchi *et al.*, 2012; Jiang *et al.*, 2016). However, the progress in this field is slow and there are essentially no interventions that improve memory reliably.

In humans, cognitive training, both early and later in life, through engagement in intellectually stimulating activities, is a powerful modulator of the brain and is associated with better cognitive functioning, as it postpones or attenuates cognitive decline, and ameliorates cognitive deficits (Wilson *et al.*, 2002; Frick & Benoit, 2010; Milgram *et al.*, 2006; Belleville & Bherer, 2012; Rebok *et al.*, 2014). Impressively, these improvements can be maintained for years (Willis *et al.*, 2006). For example, in the ACTIVE trial, a randomized multicenter trial involving cognitively normal older adults, cognitive training resulted in

improved cognitive abilities specific to the abilities trained that continued 5 years after the initiation of the intervention (Willis *et al.*, 2006).

Thus, there is sufficient evidence suggesting any activity which involves thinking and learning is beneficial when it comes to maintaining or improving brain health and preventing dementia. The more complex and challenging the mental activities, the greater benefit they offer. Also, the more brain activities and the higher frequency of enrolment, the lower is the risk for dementia. In this thesis, we will address the effects of brain tasks that can help improve and/or maintain brain health.

The effects of cognitive training could be explained by the cognitive reserve hypothesis, which rests on the ability of the brain to compensate for pathological changes associated with aging, depending on the previous stage of intellectual capability (Whalley *et al.*, 2004; Stern, 2002).

The concept of cognitive reserve originated in the late 1980s, when a study performed by Katzman *et al.*, 1988 reported that some individuals with brain changes compatible with Alzheimer's disease, clinically presented none or very little symptoms of dementia. The investigators speculated that these individuals did not show symptoms of the disease while they were alive because they started with larger brains and more neurons, and thus might be said to have had a greater "reserve" to the offset of damage and, therefore, continued to function as usual.

While passive reserve refers to brain reserves such as brain volume and the number of neurons and synapses, active reserve corresponds to the brain's potential for plasticity and reorganization of neuronal networks, allowing it to cope with or compensate for pathology (Stern, 2002; Harada *et al.*, 2013). This hypothesis supports the idea that higher levels of education, engagement in certain activities, higher socioeconomic status, and baseline intellectual capability, protect against the neuroanatomical alterations observed during the aging process (Stern, 2002). Furthermore, the concept of cognitive reserve has been put forward to account for individual differences in susceptibility to age-related brain changes (Stern, 2012). Accordingly, in humans, cognitive training induces increased dendritic length of neurons (Jacobs *et al.*, 1993), increased volumes in the HPC (Maguire *et al.*, 2006; Cannonieri *et al.*, 2007), cortical and subcortical areas (Seider *et al.*, 2016), and resulted in important changes in brain activity (Hempel *et al.*, 2004; Olesen *et al.*, 2004).

In rodents, the beneficial effects of cognitive training have also been associated with physical stimulation, which together is often referred to as environmental enrichment (EE) (Fischer, 2016). Environmental enriched rodents are typically socially housed in large groups and exposed to a variety of stimulating objects that can provide both cognitive stimulation (e.g., toys, tunnels, dwellings) and physical exercise (e.g., running wheels). For example, Vicens *et al.*, (1999, 2002) found that a water maze training task,

performed in 6-month-old mice, led to an improved performance in the water maze later at 10 and 18 months of age. More recently, Galeano *et al.* (2015) showed that life-long EE was able to rescue memory deficits in control aged rats and in aged rats that were subjected to asphyxia at birth. Additionally, several research groups have systematically shown that environmental enrichment induces synaptogenesis (Rampon *et al.*, 2000), neurogenesis (Kempermann *et al.*, 2002), cortical thickening (Mohammed *et al.*, 2002) and dendritic branching (Faherty *et al.*, 2003; Kolb *et al.*, 2003; Bindu *et al.*, 2007). Interestingly, these anatomical changes correlate with functional alterations such as increased hippocampal long-term potentiation (LTP) (Artola *et al.*, 2006, Stein *et al.* 2016). Finally, some studies assessing the physiological responses to enrichment reported variations in corticosterone levels (Schrijver *et al.*, 2002; Morley-Fletcher *et al.*, 2003; Belz *et al.*, 2003; Moncek *et al.*, 2004; Konkle *et al.*, 2010). Despite all the above-mentioned evidences, little is known about EE effects in aging animals.

Previous unpublished work in our lab has shown that in young adult rats, short cognitive training in a hole-board (HB) or the T-maze (TM) task could effectively and rapidly promote recovery from stress-induced deficits, by triggering structural, electrophysiological and ultimately functional plasticity. Importantly, this previous work also showed that, while training in the HB resulted in improved plasticity in hippocampal dependent networks and spatial memory, training in the TM improved plasticity in PFC networks and executive function, in a highly selective manner. In particular, given the heterogeneity described above, it remains to be shown if all old individuals benefit from cognitive training, or if some could perceive it as a stressor (Mohapel *et al.*, 2006), given the increased cognitive load imposed on already fragile cognitive abilities.

As already discussed, the HPC is one of the brain areas particularly vulnerable to aging (Mora *et al.*, 2007). Therefore, in this work, and bearing in mind that a specific cognitive stimulation could differently and specifically enhance a particular brain circuit, an HB food-retrieval task, designed to engage spatial reference memory (van der Staay *et al.*, 2012), was used as a cognitive-training tool in order to enhance HPC-dependent cognitive function (Van der Staay *et al.*, 1999, 2012; Depoortère *et al.*, 2010).

Considering the increased number of people over the age of 65 and the intense promotion of "cognitive exercises" as a panacea for older persons, these answers are increasingly relevant. Clearer knowledge on what delays cognitive decline and how we can maximize cognitive function is crucial to improve quality of life for these individuals.

5. Aims of the study

As the number of people over the age of 65 are increasing, an aged population is a challenge that our societies have to face. As discussed above, the cumulative effects over the lifespan, induce several structural, molecular and functional changes in the brain. Although modern medicine can fix or replace many malfunctions on our bodies, our brains are highly customized and irreversible. Therefore, the development of new therapeutic tools to tackle the maladaptive changes imposed by aging is of immediate importance. Understanding the mechanisms of aging is thus vital for this modern endeavor.

Under normal aging conditions, there is a significant variability in age-related interindividual cognitive changes, with some individuals displaying age-related cognitive decline, while others present none or minimal age-related dysfunctions. Understanding why some individuals can retain their "youth brains" while others lose cognitive ability, might be vital in an aging society. So far, evidence on the mechanisms underlying such heterogeneity are scarce.

Additionally, since memory impairments impose a substantial burden for those affected, the development of interventions to maintain and optimize cognitive functioning is of utmost importance. Hence, cognitive enrichment/training has been associated with better cognitive functioning, as it postpones or attenuates cognitive decline, and ameliorates cognitive deficits. However, current knowledge has not yet established an explicit relationship between age-related cognitive decline, cognitive training, and the remodeling of brain structures that underlie these processes.

In order to address some of the important questions that arose from human studies of aging, in this project we proposed a multi-level analysis in the rat, to explore brain aging and its modulation by cognitive training.

The present work aimed to:
- Cluster both aged and young animals by their cognitive performance in behavioral assessment as cognitively intact (good performers) and cognitively impaired (bad performers) and look for structural (dendritic morphology and volumetric alterations in specific brain regions) and molecular correlates of such differences (Chapters II and III).
- Clarify the impact of cognitive training during adulthood on the patterns of cognitive aging and its structural correlates, acknowledging the well described heterogeneity of brain aging (Chapter IV).

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Structural and molecular correlates of cognitive aging in the rat

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Structural and molecular correlates of cognitive aging in the rat

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1. Abstract

Aging is associated with cognitive decline. Herein, we studied a large cohort of old age and young adult male rats and confirmed that, as a group, older rats display poorer spatial learning and behavioral flexibility than younger adults. Surprisingly, when animals were clustered as good and bad performers, our data revealed that while in younger animals better cognitive performance was associated with longer dendritic trees and increased levels of synaptic markers in the hippocampus and prefrontal cortex, the opposite was found in the older group, in which better performance was associated with shorter dendrites and lower levels of synaptic markers. Additionally, in old, but not young individuals, worse performance correlated with increased levels of BDNF and the autophagy substrate p62, but decreased levels of the autophagy complex protein LC3. In summary, while for younger individuals "bigger is better", "smaller is better" is a more appropriate aphorism for older subjects.

2. Introduction

Aging is a process that, even in healthy individuals, is generally linked to a decline in cognitive abilities[1]. However, one of the most striking characteristics of human aging is its heterogeneity[2,3], with some individuals maintaining a preserved cognitive function until late in life. Since cognitive ability is a crucial determinant of elderly people's quality of life, a thorough understanding of the mechanisms underlying its heterogeneity is of paramount importance. To this purpose, we decided to study a large cohort of young adults (4-6-month-old) and older rats (22-24-month-old).

Rats have been intensively used as models of cognitive aging[4-7]. A large body of literature indicates that, as in humans, spatial learning and memory tasks in rodents also require the hippocampus (HPC) and the medial prefrontal cortex (mPFC), and typically display performance decrements across the lifespan[5,6,8]. In fact, older male rats, approximately 22–24 months-old, show impairments in several spatial memory tasks including: Y and T mazes[9], radial arm maze[10], Morris water maze[6-8], water radial arm maze[11] and Barnes maze[12].

The well-documented age-related behavioral deficits are concomitant, and seem to be correlated with, morphological alterations in brain structure. It is widely accepted that aging is accompanied by an overall brain volume loss, in both humans[13-16] and rats[17,18] that accompanies the decline in cognitive function. Moreover, several studies reported age-related cognitive decline to be associated with volume loss and dendritic atrophy in areas implicated in cognitive abilities, such as the HPC and the mPFC[13,18-21].

The homeostasis of the mammalian neuroarchitecture is a dynamic process involving a balance between sprouting and pruning. The mechanisms underlying these processes are particularly active during development and pathological neurodegeneration[22,23] but are also functional in physiological conditions. Of note, while in equilibrium, the relative importance of each of these processes varies throughout the lifespan, with synapse and dendritic formation generally exceeding pruning during brain development, and an opposite trend in the adult brain[23,24]. Importantly, the maintenance of this balance is under tight control through protein synthesis and autophagic recycling[23-26].

Herein, we explored cognitive aging and its structural and molecular correlates in a large set of old and young male Wistar Han rats. The results show that while for younger individuals "bigger is better", it seems that "smaller is better" is more appropriate for older subjects, as older animals with smaller dendritic trees, increased neuronal autophagy and decreased brain-derived neurotrophic factor (BDNF) and synaptic markers, presented the best performances.

3. Results
3.1. Age is associated with cognitive decline and behavioral heterogeneity

Older animals displayed a worse cognitive performance in all tested domains (working memory: $t(267)=-10.122$, $p<0.0005$, $d=1.148$; reference memory: $t(276)=-11.274$, $p<0.0005$, $d=1.403$; behavioral flexibility: $t(275)=-2.222$, $p=0.027$, $d=0.277$) (Fig. 1a,b,c). Moreover, the performance of older rats in working and reference memory was more heterogeneous than that of younger rats (working memory variance: older=290.33%^{2} vs. younger=141.12%^{2}; reference memory variance: older=262.47%^{2} vs. younger=180.47%^{2}), while no differences were observed in the distributions of behavioral flexibility performances of older and younger animals (variance: older=128.96%^{2} vs. younger=118.56%^{2}) (Fig. 1a,b,c).

Behavioral assessment
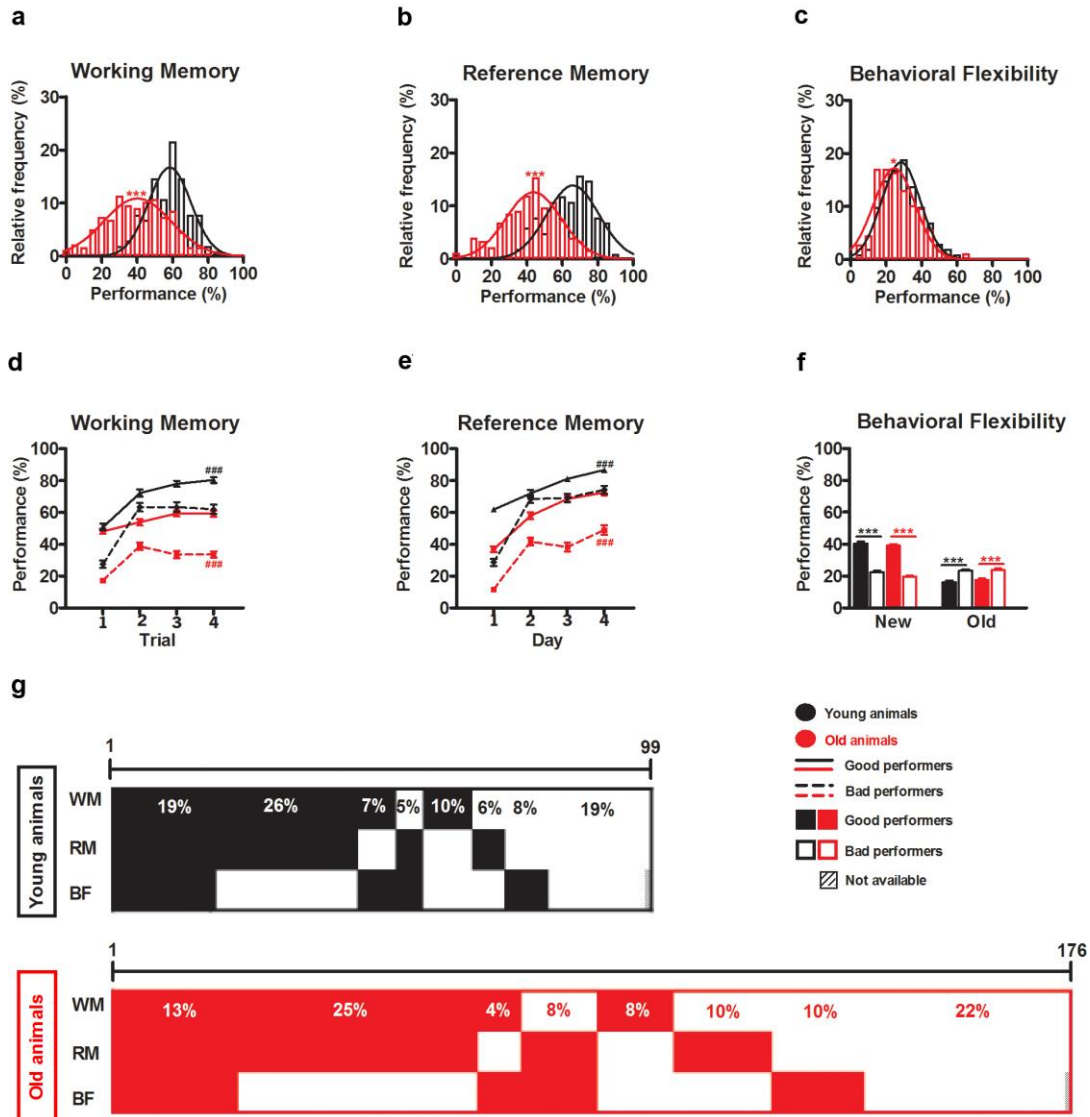
Figure 1| Behavioral assessment and performance clustering of younger and older rats.
When all animals are considered: **A)** The working memory (older n=176; younger n=102) and **B)**
reference memory (older n= 176; younger n=102) PIs of older rats are worser and broader than that of
youngsters. **C)** The performance in behavioral flexibility (older n=176; younger n=101) is less variable
but maintains the previous trend as older rats are the worst performing group. When similar age animals
are clustered (see methods for details) in Good and Bad performers: GPs and BPs clusters had
significantly different learning curves in the **D)** working memory (older: GPs n=89 BPs n=87; younger:
GPs n=63 BPs n=39) and **E)** reference memory tasks (older: GPs n=99 BPs n=77; younger: GPs n=61
BPs n=41). **F)** GPs clusters spent more time in the new and less in the old quadrants of the behavioral
flexibility task (older: GPs n=62 BPs n=114; younger: GPs n=40 BPs n=61). Interestingly, **G)** the

frequencies of the different patterns were similar in younger and older animals. Continuous lines in **A**, **B**, and **C** are Gaussian fits. Error bars represent SEM; * p<0.05; *** p<0.001.

A k-means clustering was performed to classify younger and older animals according to their performance in working memory, reference memory or behavioral flexibility tasks. This resulted, for each test and age, in two groups of subjects (good performers – GPs and bad performers – BPs), which had significantly different performances (Table 1, Fig. 1d,e,f; see also the Supplementary Figure S1 for the escape latency to find the platform (s)). A two-way ANOVA revealed a significant effect of age and performance, as a significant interaction between these two factors, both in the working and reference memory test (Table 1, Fig. 1d,e,f). Overall, in memory tests, younger adult rats performed better than older rats, with younger GPs being the best (Tukey's test p<0.001), younger BPs and older GPs intermediate and older BPs the worst performers (Fig. 1d,e). In contrast, although the performance in the behavior flexibility task was significantly different between age categories (only for the time spent in the new quadrant) and performance groups, performance of younger and older GPs was similar, as was that of younger and older BPs (Tukey's test p>0.05), without a significant interaction between age and performance group (Table 1, Fig. 1f).

A subsequent analysis of each individual's cluster membership for each of the behavioral tests revealed that these were poorly correlated, as animals were distributed across all possible combinations of GPs and BPs, without a clear predominance of impaired cognitive performance across all tests. Importantly, although cognitive performance patterns were widely variable between individuals of the same age group, for each age group, the same eight different patterns of performance were identified, and their overall distribution was approximately similar across the different ages (Fig. 1g). Of notice, the percentage of animals belonging to the GPs group in all the tests was lower in older animals when compared with younger animals (older=13%, younger=19%), while the proportion of animals belonging to BPs in all the tests was similar between the different age groups (older=22%, younger=19%).

3.2. Age triggers dendritic atrophy that correlates with individual cognitive performance

To better understand the above described behavioral differences, we analyzed the morphology of dorsal HPC neurons (dentate granular, CA3 and CA1 pyramidal neurons). Considering the granular neurons of the HPC, older animals presented shorter dendritic trees when compared to younger animals (t(37)=-8.314, p<0.0005, d=2.736, Fig. 2a). Regarding CA3 and CA1 HPC pyramidal neurons, apical dendrites

presented an age-dependent decrease in dendritic length whereas basal dendrites presented no differences in CA1 neurons but an increased dendritic length in CA3 pyramidal neurons (CA3 apical dendrite $t(37)$=-2.907, p=0.006, d=0.807; CA3 basal dendrite $t(28)$=3.968, p=0.0004, d=1.027; CA1 apical dendrite $t(38)$=-7.282, p<0.0005, d=1.914; CA1 basal dendrite $t(40)$=-1.714, p=0.094, d=0.552; Fig. 2b,c).

Morphological analysis of HPC neurons, in both young and older animals, revealed a correlation between dendritic length and the individual performances in the reference memory task. Curiously, while for younger animals the apical dendritic trees of DG and CA1 neurons presented a significant positive correlation (DG: r^2 = 0.714, p = 0.003; CA1: r^2 = 0.523, p = 0.046), in older animals this correlation was reversed, with better performers having the smallest apical trees in all three regions analyzed (DG: r^2 = -0.659, p < 0.0005; CA3: r^2 = -0.450, p = 0.027; CA1: r^2 = -0.572, p = 0.002) (Fig. 2d,h,l). We found a similar negative correlation, in older, but not younger animals, between working memory performance and dendritic tree length of DG, CA3 (apical) and CA1 (apical and basal) cells (Supplementary Table S2). Significantly, there was no correlation in older animals between behavioral flexibility performance (a more mPFC-dependent task) and dendritic length of any HPC type of cells (Supplementary Table S2; for additional information regarding individual animal performance in all cognitive tasks see Supplementary Fig. S3a,b,c). To further clarify the relationship between HPC dendritic length, age and reference memory performance, we conducted a two-way ANOVA using the clustering of animals according to performance on this test. This analysis revealed a significant effect of age, but not of performance group, and a significant interaction between the two factors, in the average dendritic length of granular neurons and the apical tree of CA1 pyramidal cells (Table 1, Fig. 2e,m). In contrast, no factor seemed to influence the length of CA1 pyramidal cell basal dendrites (Table 1).

Figure 2| Morphological analysis of HPC neuron dendritic arborizations. When a random sample of all animals is considered (older = 27; younger = 15): **A, B** and **C)** Comparison of dendritic lengths of DG granular, CA3 and CA1 pyramidal neurons between younger and older rats. **D)** Correlation between granular neuron dendritic lengths and individual performances in the reference memory task of both younger and older rats. When similar age animals are clustered (see methods for details) in Good and Bad performers according to reference memory performance (older GP = 16 (18 for CA1); older BP = 8 (9 for CA1); younger GP = 10; younger BP = 5): **E)** Average dendritic lengths for both GPs and BPs of younger and older animals. **F)** Sholl analysis of the apical dendrite of DG granular neurons. This graph presents the mean number of intersections of apical dendritic branches with consecutive 20μm spaced concentric spheres. **G)** Representative reconstructions of DG granular neurons used in the previous analysis. **H, I, J** and **K)** The same analysis was performed for CA3 pyramidal neurons and in **L, M, N**

and **O** for CA1 pyramidal neurons. Error bars represent SEM, doted lines represent confidence intervals and continuous lines are linear fits; *p<0.05; ** p<0.01; ***p<0.001. (RM – reference memory)

Regarding comparisons within age groups, aged BPs presented a significant increase in the dendritic length of both granular and apical dendrite of CA1 pyramidal neurons when compared with aged GPs (DG: t(22)=-2.632, p=0.015, d=1.033; CA1 apical dendrite: t(25)=-3.312, p=0.003, d=1.382) (Fig. 2e,m). Also, regarding granular neurons, a significant difference was observed between young GPs and BPs. Here, GPs display higher dendritic lengths when compared with BPs (t(13)=3.540, p=0.004, d=1.952) (Fig. 2e). Data on CA3 pyramidal neurons revealed a significant effect of age, but not of performance group nor any interaction, in the length of both basal and apical dendrites (Table1, Fig. 2i). To explore in which parts of the dendritic tree laid the above-mentioned differences, we performed a Sholl analysis, which measures the number of intersections as a function of distance from the soma. Results for granular dendrites revealed a significant effect of age, but not of performance group, and a significant interaction between the two (Table 1, Fig. 2f). Repeated measures ANOVA revealed that younger GPs, when compared with the BP group, had an overall increase in the number of intersections ($F_{(1,13)}$=11.050, p=0.005, $\eta\rho^2$=0.459), both proximally and distally (Fig. 2f). Results of the Two-way ANOVA analysis for CA3 and CA1 apical dendrites revealed no significant effect of age, performance, neither an interaction between these two factors (Fig. 2j,n). However, group comparisons revealed an overall increase in the number of intersections in apical CA1 dendrites of young GPs when compared with the BP group ($F_{(1,13)}$=6.294, p=0.026, $\eta\rho^2$=0.326) (Fig. 2n). These alterations observed in HPC neurons are exemplified in the reconstructions of figures 2g,k,o.

Since some of the cognitive tasks assessed in this work were mPFC-dependent, we also analyzed the morphology of mPFC neurons (Cg/PL and IL pyramidal neurons; Supplementary Fig. S4). We found an age-dependent reduction in the length of basal dendrites of Cg/PL pyramidal neurons, but no major changes in other dendritic domains (Supplementary Fig. S4a,b). Pearson correlations between behavioral performance and dendritic length showed a significant association between working memory and the apical dendritic length of Cg/PL, that was positive in younger subjects (r^2= 0.630, p = 0.012) and negative in older animals (r^2 = -0.504, p = 0.007) (Supplementary Fig. S4c). The performance in the reference memory task was only significantly negatively correlated with the apical dendritic length of IL pyramidal neurons of older animals (r^2 = -0.465, p = 0.029) (Supplementary Fig. S4o). Regarding the behavioral flexibility task, only in younger animals a positive correlation was found between this task and the apical dendritic length of IL pyramidal neurons (r=0.611, p=0.046; Supplementary Table S5; for additional

information regarding individual animal performance in all cognitive tasks see Supplementary Fig. S3e,f). In addition to individual correlations, we performed within group comparisons (older and younger GPs and BPs for each task) on average dendritic lengths and distribution of dendritic processes. Interestingly, differences were only present between IL neurons of GPs and BPs in younger animals (t(10)=2.377, p=0.039, d=1.550) (Supplementary Fig. S4p). Finally, the distribution of dendritic processes resulting from Sholl analysis in mPFC neurons showed a significantly more ramified apical dendritic tree of IL pyramidal neurons in younger GPs as compared with younger BPs ($F_{(1,10)}$=9.339, p=0.012, $\eta\rho^2$=0.483), with no differences in the other parameters (Supplementary Fig. S4q). For a comprehensive overview of the simultaneous alterations occurring at different brain regions, Supplementary Fig. S6 depicts the individual morphological alterations of the analyzed animals, including animal performance and respective relative dendritic lengths of DG, CA3, CA1, Cg-PL and IL brain regions.

3.3. Impaired autophagy impacts on dendritic structure

Autophagic activity has been identified as a critical mechanism underlying dendritic remodeling[25]. To test the hypothesis that autophagy signaling is disrupted in aging and could be associated with alterations in dendritic recycling in the HPC, we performed western blot analysis of the autophagosome markers LC3 and p62. To determine the relationship between autophagic activity and dendritic size, protein levels of BDNF were analyzed. As for structural markers of synaptic function the levels of PSD95, SNAP25 and SYP were determined (Fig. 3c).

To test the association between dendritic length and autophagy/neurotrophin levels, the levels of the synaptic marker PSD95 (a surrogate marker of dendritic extension) was correlated with both p62 (whose increased levels represent decreased autophagic activity) and BDNF. In both younger and older animals, HPC levels of PSD95 were positively correlated with p62 (younger: r=0.850, p=0.001; older r=0.878, p<0.0005; Fig. 3j) and BDNF (younger: r=0.671, p=0.024; older r=0.692, p=0.001; Fig. 3k).

Western blot analysis - Dorsal HPC
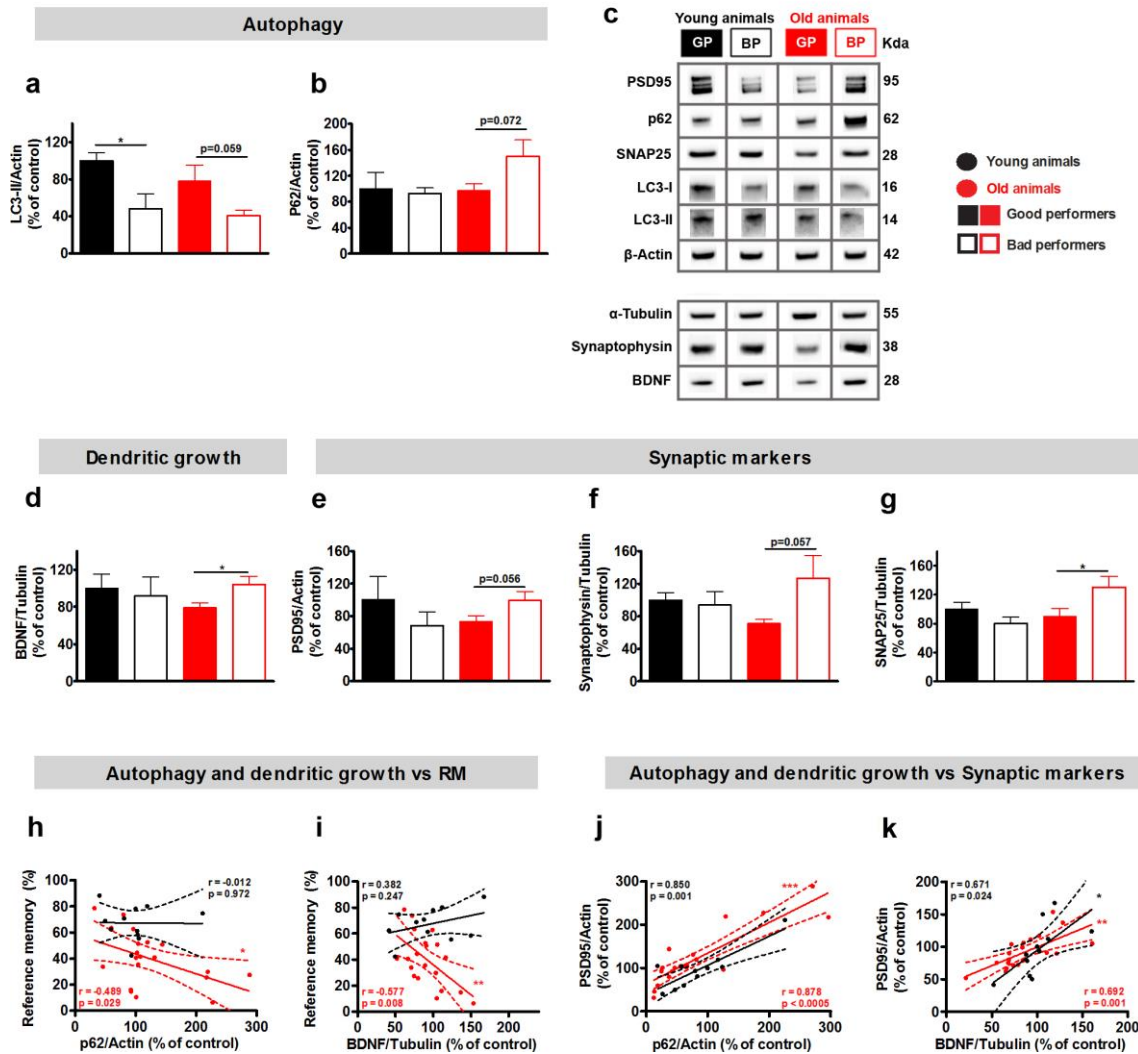
Figure 3| Defective autophagy signaling and dendritic pruning in the HPC of older BPs.
Performance in reference memory was used to cluster (see methods for details) both younger and older animals as GPs and BPs. A random sample of these were used for molecular analyses (younger GPs = 5-6; younger BPs = 5; older GPs = 10; older BPs = 9-10). **A** and **B)** Levels of autophagy markers, LC3-II **(A)** and p62 **(B)**, normalized to actin. **D)** BDNF levels normalized to tubulin. **E, F, G)** Levels of synaptic markers PSD95, SYP, and SNAP25 normalized to actin, tubulin, and tubulin, respectively. **C)** Representative western blots of PSD95, p62, SNAP25, LC3, Actin, Tubulin, SYP, and BDNF. For each protein, the blots were cropped from different parts of the same gel. **H** and **I)** Correlation between RM performance and p62 or BDNF levels, respectively. **J** and **K)** Correlation between PSD95 and p62 or BDNF levels, suggesting a relationship between the levels of synaptic markers and autophagy or dendritic growth, respectively. Error bars represent SEM, doted lines represent confidence intervals and continuous lines are linear fits; $*p<0.05$. (RM – reference memory)

Two-way ANOVA revealed a significant effect of reference memory performance, but not of age, neither an interaction between them, in the HPC levels of LC3-II, but not of p62 (Fig. 3a,b; Table 1). Group comparisons further revealed that, in younger animals, the levels of LC3-II in the HPC were significantly lower in BPs than in GPs (t(9)=2.988, p=0.015, d=1.750; Fig. 3a), while the levels of p62 in the HPC were similar between the two groups. In older animals there was a trend toward BPs animals having less HPC LC3-II (t(18)=2.020, p=0.059, d=0.903); Fig. 3a) and more p62 (t(18)=-1.912, p=0.072, d=0.855; Fig. 3b). At the individual level, younger animals had a significant positive correlation between performance in the reference memory task and the level of HPC LC3-II (r=0.790, p=0.004), but not of p62, while in older rats there was a significant correlation between reference memory performance and the levels of both HPC LC3-II (r=0.523, p=0.018) and p62 (r=-0.489, p=0.029; Fig. 3h) (see also Supplementary Table S7). There were no significant correlations between any HPC autophagy marker and performance in the working memory task or behavioral flexibility tasks (Supplementary Table S7).

Hippocampal BDNF protein levels were similar in younger BPs and GPs groups, but were significantly higher in older BPs than in older GPs (t(18)=-2.500, p=0.022, d=1.118; Fig. 3d). In younger animals, there was also a trend toward a positive correlation between HPC BDNF levels and performance in working memory task (r=0.610, p=0.061) but not in the reference memory task (r=0.382, p=0.247; Fig. 3i) nor in the behavioral flexibility task (Supplementary Table S7). In older rats, HPC BDNF levels were significantly negatively correlated with the performance in reference and working memory tasks (reference memory: r=-0.577, p=0.008; Fig. 3i; working memory: r=-0.447, p=0.048) but not the behavioral flexibility task (Supplementary Table S7).

Regarding synaptic markers, only HPC SNAP25 levels (Fig. 3g, Table 1) had a significant interaction between age and performance group, without significant effect of either factor alone. In addition, HPC levels of PSD95 (Fig. 3e), SYP (Fig. 3f) and SNAP25 (Fig. 3g) were different between GPs and BPs groups solely in older animals. In line with data from BDNF, a significant increase was observed in the levels of SNAP25 and a trend towards an increase of PSD95 and SYP in older BPs, when compared with older GPs (SNAP25 t(18)=-2.192, p=0.042, d=0.980; PSD95 t(18)=-2.045, p=0.056, d=0.914; SYP t(17)=-2.042, p=0.057, d=0.912). Lastly, concerning older animals, HPC PSD95 levels showed a significant negative correlation with reference (r=-0.448, p=0.048) and working memory (r=-0.513, p=0.021) performances, while HPC SNAP25 levels presented solely a significant negative correlation with the performance in the reference memory task (reference memory task: r=-0.560, p=0.010, working memory task: r=-0.339, p=0.143). For HPC SYP levels, a trend toward a negative correlation with reference memory (r=-0.453, p=0.052) and working memory tests (r=-0.406, p=0.085) was observed. No

correlations were found between synaptic markers and the performance in the behavioral flexibility task (Supplementary Table S7; for additional information regarding individual animal performance in all cognitive tasks see Supplementary Fig. S3d).

As previously described for the HPC, we performed an analysis of autophagic- and dendritic growth-related proteins in the mPFC (Supplementary Fig. S8). When animals were grouped by performance in the working memory, within group analysis revealed that in younger animals, levels of LC3-II and p62 were similar between GPs and BPs whereas in older BPs, there was a significant decrease in the levels of LC3-II (t(16)=2.197, p=0.043, d=1.045) and a significant increase in the levels of p62 (t(17)=-3.326, p=0.004, d=1.515), suggesting a lower level of autophagy in older BPs (Supplementary Fig. S8a,b). Also, older animals, but not younger animals, presented a negative correlation between p62 and the performance in reference (r=-0.504, p=0.028) and working memory task (r=-0.646, p=0.003; Supplementary Fig. S8h), but not the behavioral flexibility task (Supplementary Table S9). Regarding neurotrophins and synaptic markers, mPFC BDNF, PSD95 and SYP protein levels were significantly higher in older BPs compared with older GPs (BDNF t(18)=-2.226, p=0.039, d=0.995; PSD95 t(16)=-1.959, p=0.068, d=0.937; SYP t(18)=-2.108, p=0.049, d=0.943) (Supplementary Fig. S8d,e,f). Also, BDNF levels were not correlated with performance in the working memory (Supplementary Fig. S8i), reference memory or behavioral flexibility (Supplementary Table S9) in any age group, and were correlated with PSD95 in older animals (r= 0.671, p=0.002; Supplementary Fig. S8k) but not younger rats (r=-0.256, p=0.476; Supplementary Fig. S8k; for additional information regarding individual animal performance in all cognitive tasks see Supplementary Fig. S3g). This data is in agreement to what we previously described for the HPC, further showing that the levels of autophagy in mPFC neurons are strongly and positively related with dendritic pruning and better performances in older individuals.

4. Discussion

The present study addressed the heterogeneity of cognitive-aging from a multidimensional perspective to reveal a hitherto of unappreciated complexity. It explored its underpinnings, highlighting, for the first time, the role of the balance between neurotrophic and autophagic activities in such processes. Data herein presented confirmed that, despite a general aging-associated cognitive decline, the performance of both young adult and older rats in working and reference memory tasks is heterogeneous, particularly in older individuals[2,35]; in the latter, we confirmed that a certain proportion of subjects maintain spatial memory abilities comparable to those of younger animals[6,7]. Of notice, the data clearly revealed, for the first time,

that this age-associated increase in the dispersion of individual performance is not universal, as it was not present in all cognitive dimensions (e.g. the behavioral flexibility task).

The working and reference memory tasks both assess spatial learning and memory, albeit within a different timeframe: while the former is dependent on short-term memory and HPC to mPFC connections[29,36], the latter depends on long-term memory and largely on the integrity of the HPC[28]. In contrast, the behavioral flexibility task is memory independent and assesses the ability to adapt to changing circumstances[37], a critical component of executive function, which is a very distinct cognitive ability. In light of this distinction, the observation that aging is accompanied by an increased inter-individual heterogeneity (and a performance decline, on average) in memory-dependent but not executive-function-dependent tasks adds yet another layer of heterogeneity to the aging process, and strongly suggests that some cognitive functions (and the networks sub-serving them) are more prone to aging than others. Significantly, this seems also to be the case in humans[38] in which both long-term and working memory are more influenced by age-related impairments than knowledge of vocabulary and priming, a form of non-declarative memory. Despite this, the herein reported relative preservation of executive function in rodents might seem to contradict several studies in humans showing that executive processes are also disrupted in aging[39-41]. However, it is important to highlight that, besides the obvious species difference, executive function tasks in humans are often contaminated by deficits in speed of processing[42] which are well known to be affected by aging. On the contrary, in our experiments, not only was average swimming speed similar in all older subjects (and not significantly different from that of their younger counterparts) but also the behavioral flexibility test is independent of it.

In addition to the two layers of heterogeneity in aging discussed above, analysis of each animal's membership to either the GPs or BPs group, for each behavioral test, further revealed another dimension of inter-individual heterogeneity. Indeed, when comparing cluster membership for each individual in each test, we showed for the first time that most animals were GPs in some tests and BPs in others, without a clear separating pattern in either younger or older groups. Moreover, the overall distribution of animals according to their group membership (GPs or BPs) for the 3 tests (working and reference memory and behavioral flexibility) was strikingly similar in both age groups, with only 13% of older and 19% of younger animals being good in every task and 22% of older and 19% of younger animals being bad in every task. More importantly, this third heterogeneity level seems to be independent of the other two. In other words, despite all the inter-individual heterogeneity (within a given test) and the heterogeneity between tests (with reference and working memory being more sensitive to aging than behavioral flexibility) there is also heterogeneity, at the individual level, as to being a GP or BP for each behavioral test.

In summary, the behavioral data suggest that cognitive decline in aging is not inevitable, or strictly linked to chronological age and that, even in a relatively homogeneous population of animals such as the one in this study, there is a high variability and complexity in the way the different cognitive functions are preserved/impaired in each individual. Understanding the underpinnings of individual differences may help to explain the observed heterogeneity and, possibly, what determines the existence of healthier agers, which was next studied at the morphological and molecular levels.

Changes in the morphology of neuronal dendritic trees were shown to correlate with cognitive alterations in both rodents and humans[43,44]. In aging, it is already well established that memory impairments are not related with neuronal loss[35,45] but rather to volume changes[17] and altered morphology of neuronal dendritic trees[46,47]. In the present work, using a large number of rats (15 young and 27 old) we showed that, on average, older animals have shorter apical dendritic arborizations in dorsal HPC neurons (dentate gyrus granules and CA1 and CA3 pyramids) but similar apical dendritic trees in mPFC neurons (Cg/PL and IL layers II/III pyramids) when compared to younger animals. These findings are in line with most previous studies that analyzed one or the other region (HPC[48-53]; mPFC[54-56]) and might suggest that the frontal regions might be less affected by the aging process or that age-related changes in neuronal morphology appear later in the mPFC. This is partly corroborated by the fact that age-related apical dendritic retraction in the mPFC was only reported in one study[57]. Interestingly, this relative mPFC "resilience" might be specific for the superficial layers, since deeper, layer V, pyramidal neurons, similar to hippocampal cells, exhibit age-related apical dendritic retraction at 20-22 months[20,53,56]. Importantly, this has been observed in humans, in which age-related dendritic retraction, in the same individuals, was 3 times more prominent in the deep than in the superficial PFC pyramids[58]. The fact that mPFC dendrites are less affected by aging fits perfectly with the behavioral data presented here, pointing to an attenuated age-associated decline of executive functions.

Another major novelty of the present work is the finding that older animals with deficits in HPC-dependent tasks have larger dendritic trees in the HPC than cognitively intact rats of the same age. Some previous papers had already shown that hippocampal cells from older rats[59] and older humans[60,61] had increased dendritic length, but in none of these studies were subjects cognitively characterized. Interestingly, in the mPFC, despite no overall age-related retraction, there was a similar, albeit with smaller magnitude, association between bigger apical dendritic trees in layer II/III Cg/PL pyramids and worse performance in the working memory (mPFC-dependent) test. This association, in older animals, between larger dendritic trees and poorer cognitive function might be considered contra-intuitive. However, while bigger dendritic trees might mean more connectivity and better neuronal function, it is also well described that

the accumulation of "waste" dendritic material and large dendritic trees, for example as a result of impaired autophagy and dendritic pruning deficits, hampers neuronal function and correlates with cognitive deficits, in both humans and animals[23]. Interestingly, in Fragile-X-syndrome patients, who have impaired dendritic pruning, there is also an inverse correlation between hippocampal volume and cognitive performance, which is not present in age-matched individuals without pruning deficits[62]. Of note, in the present work, the inverse correlations between dendritic tree length and cognitive performance are not present in the group of younger adults (in which an opposite trend is observed), supporting an age-related phenomenon. Together, these results suggest that the inverse correlation between large dendritic trees and poor cognitive performance in the elderly, might be attributed to age-associated dendritic pruning deficits leading to larger, less efficient, dendritic trees. In light of this hypothesis, individual differences in dendritic pruning might also underpin the individual heterogeneity in cognitive aging.

Dendritic pruning is a mechanism often used to selectively remove unnecessary and exuberant neuronal branches, not only in the immature nervous system[23] but also in the adult HPC[63], thus ensuring the proper formation of functional optimized circuitries. Dendritic and synaptic pruning is highly dependent on autophagy-dependent protein turnover, as animals presenting constitutional[23] or induced[64] inhibition of autophagy have larger dendritic trees and increased spine density, which correlate with cognitive deficits. In order to further dissect whether this could contribute to the observed morphology, we analyzed the levels of autophagic activity in the HPC and mPFC. Furthermore, this was complemented with a quantification of the neurotrophin BDNF, a main inducer of dendritic and spine growth[65]. Finally, given the technical challenge to assess protein levels and dendritic tree length in the same region of the same animals, these results were correlated with pre- and post-synaptic markers. Indeed, there is a consensus in the literature that these levels, particularly when concordant, are a good surrogate of synaptic abundance and dendritic tree complexity[66-68]. In support of this assumption, here we show that older, but not young, cognitively impaired animals have higher levels of these synaptic proteins than age-matched cognitively intact rats, precisely replicating the findings from the dendritic tree analysis.

With the present work, we reveal that older cognitively impaired animals have reduced autophagic activity in both the dorsal HPC and the mPFC, when compared with older cognitively intact rats. Of notice is the fact that a decrease in the relative abundance of the autophagic vacuole marker LC3-II (lipidated LC3) was accompanied by a correspondent increase in the relative abundance of the autophagy cargo-protein p62[69], attesting the robustness of the findings. Significantly, these observations were specific for older animals, as levels of autophagic markers in both brain regions did not differ between cognitively intact and cognitively impaired younger adult individuals. In most organisms, pathological aging is associated

with decreased autophagic activity and autophagy inhibition induces degenerative changes that resemble those associated with aging[70]. While the mechanisms of such relationship are far from being well understood, the most prevalent hypothesis considers a failure to clean toxic/waste protein debris, that accumulate with time and induce cellular dysfunction[70]. In line with this, we found that, in the older, decreased levels of autophagy are strongly and inversely correlated with the abundance of synaptic markers, a surrogate marker of dendritic length. Our findings, however, further extend the interpretation of the previous observations, by suggesting that in neurons, decreased autophagy results in less dendritic pruning and an accumulation of dendrites that hamper neuronal function. Significantly, this does not seem to be an inevitable consequence of aging, as levels of autophagic and synaptic markers in older cognitively intact individuals were strikingly similar to those of younger animals. Of note, BDNF levels similarly did not vary significantly with aging but were also increased in older cognitively impaired, compared with cognitively intact animals, suggesting that increased dendritic growth, as well as decreased autophagic activity, might also contribute to the increased dendritic length observed in these animals.

Many factors could induce a decreased autophagic activity, similar to that presented by older cognitively impaired individuals. One of the best candidates is an enhanced activity of the mechanistic target of rapamycin (mTOR, formerly known as mammalian target of rapamycin mTOR) complex, a redox/energy/nutrient sensor that inhibits autophagy and stimulates protein synthesis[71]. Increased mTOR activity (resulting in decreased autophagic activity) has been linked to cognitive dysfunction and learning deficits in a variety of disorders[72,73], which are also associated with an increase in dendritic spines[74]. More importantly, and in line with our results, lifelong treatment of mice[75,76] or accelerated senescence rats[77] with the mTOR inhibitor rapamycin (that is considered an autophagy inducer) improved age-related cognitive dysfunction. Given the above, it is plausible to conclude that an impairment of neuronal autophagic activity could result in a scarcity of pruning mechanisms in aging neural circuits, leading to an accumulation of dendritic material and to the consequent decrease of cognitive performance.

Other factors that are commonly associated with aging could also impact dendritic length, including altered glutamatergic transmission and insulin signaling. However, since these would ultimately lead to changes in neurotrophins and/or autophagic processes, we did not address these separately in the present work. Nevertheless, in order to gain full insight into the individual determinants of altered autophagic activity, these and other factors should be taken in consideration. Furthermore, insights into the relevance of all these mechanisms can only be obtained by experimental manipulations of autophagy, which were not the scope of the present work but should be pursued in the future.

In conclusion, our findings show that alterations in the dendritic length of neurons are associated with the heterogeneity observed in the performance of young and older animals, with a twist. Indeed, it seems that while for younger animals "bigger is better", for older animals "smaller is definitely better". Moreover, we herein provide evidence that, in older animals, dendritic length differences and behavioral heterogeneity can be ascribed to variations in neurotrophin levels and, more importantly, autophagic activity. To summarize these associations, we propose a model where the balance between neurotrophins and autophagic activity regulates dendritic growth/pruning thus contributing to the heterogeneity in the cognitive function of younger vs older animals (Fig. 4). This data represents a paradigm shift in understanding the individual differences observed with aging.

Figure 4| Schematic representation of the relations between age, cognitive performance, neuronal morphology, autophagy, synaptic and dendritic growth markers, in the HPC and mPFC. In younger animals "bigger is better"; GPs have the biggest dendritic trees. However, there are no extensive differences in autophagy levels (LC3-II, p62), dendritic growth (BDNF) and synaptic markers (PSD95, SNAP25, SYP). In older animals, it seems that "smaller is better". BPs have the bigger dendritic trees, associated with a decrease in the levels of autophagy (LC3-II, p62), and an increase in dendritic growth (BDNF) and synaptic markers (PSD95, SNAP25, SYP).

5. Methods
5.1. Animals

All procedures were carried out in accordance with local regulations (Decreto-Lei n.º 113/2013) and European Union Directive 2010/63/EU on animal care and experimentation. Animal facilities and the people directly involved in animal experiments were certified by the Portuguese regulatory entity – DGAV (Direção-Geral de Alimentação e Veterinária). All protocols were approved by the Ethics Committee of the Life and Health Sciences Research Institute (ICVS). All the male Wistar Han rats (Charles River Laboratories, Barcelona, Spain) used in the study were housed in groups of 2 and maintained under standard laboratory conditions: artificial 12h light/dark cycle (lights on from 08:00a.m. to 08:00p.m.); room temperature 22°C; *ad libitum* access to food and water. A total of 176 old (22-24-month-old) and 102 younger (4-6-month-old) male rats were used in the study. The animals were tested in a battery of water-maze based tests to assess cognition. The brains of a randomly selected subset of both younger and older animals were subjected to morphological (3D neuron reconstruction) analyses and of another randomly selected subset to molecular analyses (western blot). The remainder animals were sacrificed at various time points for several other analyses not included in the present study. All behavioral testing was conducted during the light phase of the daily light cycle.

5.2. Behavioral assessment

The cognitive status of all the animals was assessed based on performance in a series of tasks using the water maze. Animals were tested during 8 days in 3 tests designed to assess different cognitive domains: spatial working memory, reference memory and behavioral flexibility[27]. The apparatus consisted of a large circular black pool (170cm diameter), filled to a depth of 31cm with water (at 22°C), which was divided by imaginary lines in 4 equal-sized quadrants. During the execution of the test, a submerged cylindrical black platform (12cm diameter, 30cm high) was hidden below the water surface at the center of one of the quadrants. The room was dimly lit and extrinsic visual clues were glued to the walls surrounding the tank and kept unaltered during the duration of the experiment. Data was collected using a video camera placed above the center of the pool connected to a video-tracking system (Veiwpoint, Champagne au Mont d'Or, France).

Working memory task: this task is a variation of the spatial reference memory test[28] and depends on mPFC function[29]. Its goal is to assess the ability of rats to learn the position of a hidden platform and to keep this information online during 4 consecutive trials. This test consisted of 4 days of acquisition in which the position of the platform was kept constant during each daily trial (with a maximum of 120s per

trial) but was altered to a different quadrant every changing day (such that all four quadrants were used). Thus, the animal cannot know where the platform was hidden on trial 1 of each day. Test sessions begun with rats facing the wall of the maze, being placed at one of the four different starting points (north, east, south or west) which were different every day. A trial was considered complete once the escape platform had been reached by the rat. The time spent to reach the platform was recorded. Animals were then allowed to spend 30s in the platform, after which they were towel-dried and allowed to rest in a holding cage some s before being returned to the maze. When the escape platform was not reached within 120s, the experimenter guided the animal to the platform and an escape latency of 120s was recorded.

Reference memory task: this task is a HPC-dependent task[28] that evaluates the ability of the animal to learn the location of a hidden platform during 4 consecutive days – spatial reference memory. After the working memory procedure (e.g. on days 5-7), the platform remained in the same quadrant as on day 4 and animals were tested for an additional 3 days without changing the location of the hidden platform. The remaining procedures were like those described above.

Behavioral flexibility task: this is a mPFC-dependent task and was performed after the reference memory task (day 8). For this test, the escape platform was moved and located in the opposite quadrant of where it had been for the previous 4 days. All the procedures were similar those described above. For this task, time spent swimming in each quadrant was recorded and analyzed.

5.3. Histological procedures

Two months after the behavioral evaluation, 50 older (22-24-month-old) and 27 younger (4-6-month-old) rats were randomly selected, deeply anesthetized with sodium pentobarbital and perfused with saline for Golgi-Cox staining (older n=30, younger n=15) and western blot analysis (older n=20, younger n=12).

Golgi-cox staining: After perfusion, the brains were removed, immersed in 25mL of Golgi-Cox solution[30] (1:1 solution of 5% potassium dichromate and 5% mercury chloride diluted 4:10 with 5% potassium chromate) and kept in the dark at room temperature for 14 days. Brains were then transferred to a 30% sucrose solution. At this moment, brains were stored in the dark at 4°C from a minimum of 3 days to a maximum of 2 months, before being cut on a vibratome. Coronal sections (200μm thick) were collected in 6% sucrose and blotted dry onto cleaned, gelatin-coated microscope slides. Subsequently, sections were alkalinized in 18.7% ammonia, developed in Dektol (Kodak), fixed in Kodak Rapid Fix, dehydrated through a graded series of ethanol of increasing concentrations and cleared in xylene before being covered

in mounting media (Entellan New) and coverslipped. The slides were stored in the dark and exposed to the air, at room temperature, until being analyzed.

5.4. Structural analysis

To ensure an unbiased analysis, slides were re-coded by the lab technician (not involved in the research) as soon as they were prepared and all neuronal reconstructions were done blind to animal age or performance group. To minimize bias, codes were only broken after all data was collected and entered into the database.

Dendritic tree analysis: Dendritic arborizations were analyzed in the dentate gyrus (DG), cornus ammonis 3 (CA3) and cornus ammonis 1 (CA1) regions of the dorsal HPC, and in layer II/III of the cingulate/prelimbic (Cg/PL) and infralimbic (IL) areas of the mPFC. The dorsal/ventral HPC division was performed according to Pinto et al. (2015)[31] and the identification of layer II/III of the Cg/PL and IL areas was achieved according to Cerqueira et al. (2007b)[32].

The granular neurons of the DG were readily identified based on their round cell bodies, which are located in the stratum granulosum of the suprapyramidal and infrapyramidal blades. Pyramidal neurons from the HPC (CA1 and CA3) or the mPFC (Cg/PL and IL) were readily identified by their characteristic triangular shaped soma. All neurons were chosen for reconstruction based on the criteria described by Uylings et al. (1986)[33]: i) full impregnation of the neurons along the entire length of the dendritic tree; (ii) apical dendrite without truncated branches, except on the most superficial layer; (iii) presence of at least 3 primary basal dendritic shafts, each of which branched at least once (when applicable); (iv) relative isolation from neighboring impregnated cells that could interfere with analysis (clear somatic boundaries) (v) no morphological changes attributable to incomplete dendritic impregnation of Golgi-Cox staining. To minimize selection bias, slices containing the region of interest were randomly searched and the first 5-10 neurons fulfilling the above criteria (maximum of 3 neurons per slice) were chosen. For each selected neuron, all branches of the dendritic tree were reconstructed at 600× magnification, using a motorized microscope (Olympus BX51 Microscope with oil-objectives), attached to a camera (QImaging® Retiga-2000R digital camera, Surrey, Canada) and the Neurolucida software (Microbrightfield, VT, USA). A 3-D analysis of the reconstructed neurons was performed using NeuroExplorer software (Microbrightfield). Dendritic morphology was examined by assessing the total dendritic length and the number of dendritic branches. In addition, to assess differences in the arrangement of dendritic material, a 3-D version of a Sholl analysis[34] was performed. For this, the number of intersections of dendrites with concentric spheres positioned at radial intervals of 20 μm from the soma was recorded.

5.5. Western blot analysis

Rat brain tissue (dorsal HPC and mPFC) was lysed with 1X RIPA buffer supplemented with protease inhibitors (cOmplete Mini EDTA-free, Roche, Basel, Switzerland) and phosphatase inhibitors (phosphatase inhibitor Cocktail 2 and 3, Sigma-Aldrich, St. Louis, Missouri, US). Homogenization was performed with an electric homogenizer, and homogenates were maintained in constant agitation for 2h at 4°C. After that, homogenates were centrifuged at 12000rpm at 4°C for 20min and supernatants were collected for western blotting. Protein concentration was determined using the Bradford assay (Bio-Rad, Hercules, CA, USA). Twenty micrograms of total protein were loaded into SDS-Page gels and then transferred to nitrocellulose membranes. The membrane was stained with Ponceau to verify successful transfer. Membranes were incubated with the following primary antibodies: microtubule-associated protein 1A/1B-light chain 3 (LC3) (1:1000, Cell signaling, Danvers, US), nucleoporin 62 (p62) (1:1000, Novus, St. Charles, US), BDNF (1:1000, Abcam, Cambridge, UK), synaptosomal-associated protein 25 (SNAP25) (1:5000, Abcam), synaptophysin (SYP) (1:10000, Abcam), postsynaptic density protein 95 (PSD95) (1:1000, Abcam), α-tubulin (1:5000, Sigma-Aldrich, St. Louis, Missouri, US), and β-actin (1:1000, Ambion, Naugatuck, US), overnight at 4°C. Antibody affinity was detected by chemiluminescence (ECL Bio-Rad). Band quantification was done using ImageLab 4.1 (Bio-Rad) using α-tubulin or β-actin as the loading control. Full-length images are presented in Supplementary Fig. S10.

5.6. Statistical analysis

All statistical analysis was conducted in the SPSS software package version 19 (IBM corporation, Armonk, New York, US). After confirmation of homogeneity and normality, appropriate statistical tests were applied to the data. To facilitate direct comparisons between different tests, results of all behavioral tests were converted to a 0-100% scale, where 0% indicates worst possible performance (120s to reach the platform for the working and reference memory tests or no time in target quadrant for the behavioral flexibility task) and 100% indicates best possible performance (0s to reach the platform or total time in the target quadrant). Also, in order to allow individual correlations between the performance of the animals in the working and reference memory tests, which consist of several testing days, and structural parameters, performance index (PI) was calculated for each test by employing the following formula: $PI=(P1+(P3+P4)/2)/2$ where Pn represents the average performance of each animal on trial n (for working memory) or day n (for reference memory); importantly, the index value can be directly read as the average performance of each animal per trial/day. Regarding the behavioral flexibility test, the

percentage of time spent in the target quadrant (performance index) was used to assess the individual correlations. Clustering of animals in good and bad performance groups was done using the k-means cluster analysis according to their performance index in the working memory, reference memory or behavioral flexibility tasks. The distance between cluster centers of the two desired groups was maximized in an iterative process (maximum number of iterations set to 25). Comparisons between two groups were done using the two-tailed t-test and repeated measures ANOVA. Two-way ANOVA was used to evaluate the impact of age and the effect of group performance in further behavioral, structural and molecular data. For multiple comparisons in the behavioral tasks one-way or repeated measures ANOVA were used. Differences between groups were then determined by Tukey´s honestly significant difference test post-hoc analysis. Pearson correlations were computed between continuous variables. Measures of effect size (Cohen's d, Eta-squared or Pearson correlations) are presented whenever appropriate. Note that, regarding the number of younger animals that performed the behavioral flexibility task, only 101 out of the102 animals were included in the analysis; this was due to tracking problems. Results are expressed as group mean ± SEM. Differences were considered significant if $*p<0.05$; $**p<0.01$; $***p<0.001$.

6.   References

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Table 1| Results of repeated measures, *t*-test and two-way ANOVA on the data obtained from younger and older animals.

Repeated measures

Behavioral assessment (Fig. 1d,e,f)

Working Memory *(number of GP, BP)*

	df	F	P	ηρ²
Older animals (GP n= 89, BP n=87)	1,174	**236.373**	**<0.0005**	**0.576**
Younger animals (GP n= 63, BP n=39)	1,100	**97.730**	**<0.0005**	**0.494**

Reference Memory *(number of GP, BP)*

	df	F	P	ηρ²
Older animals (GP n= 99, BP n=77)	1,174	**181.672**	**<0.0005**	**0.511**
Younger animals (GP n= 61, BP n=41)	1,100	**79.790**	**<0.0005**	**0.444**

t-test

Behavioral Flexibility - New Quadrant (number of GP, BP)

	df	t	P	d
Older animals (GP n= 62, BP n=114)	**174**	**-18.886**	**<0.0005**	**2.882**
Younger animals (GP n= 40, BP n=61)	**99**	**13.685**	**<0.0005**	**2.738**

Behavioral Flexibility - Old Quadrant (number of GP, BP)

	df	t	P	d
Older animals (GP n= 62, BP n=114)	**158**	**5.047**	**<0.0005**	**0.761**
Younger animals (GP n= 40, BP n=61)	**99**	**-5.217**	**<0.0005**	**1.063**

Two-way ANOVA

Behavioral assessment (Fig. 1d,e,f)	df	Performance			Age			Interaction		
	df	F	P	ηρ²	F	P	ηρ²	F	P	ηρ²
Working Memory	1,274	**273.651**	**<0.0005**	**0.500**	**245.073**	**<0.0005**	**0.472**	**10.753**	**0.001**	**0.038**
Reference Memory	1,274	**213.618**	**<0.0005**	**0.438**	**238.943**	**<0.0005**	**0.466**	**10.141**	**0.002**	**0.036**
Behavioral Flexibility										
New quadrant	1,273	**499.625**	**<0.0005**	**0.647**	**6.291**	**0.013**	**0.023**	0.823	0.365	0.003
Old quadrant	1,273	**42.565**	**<0.0005**	**0.135**	0.738	0.391	0.003	0.153	0.696	0.001
Morphological analysis - Hippocampus (Fig. 2e,i,m)										
Granular Neurons	1,35	3.276	0.079	0.086	**67.480**	**<0.0005**	**0.658**	**22.030**	**<0.0005**	**0.386**
CA3 pyramidal neurons (apical tree)	1,36	2.513	0.112	0.065	**4.784**	**0.035**	**0.117**	0.183	0.671	0.005
CA3 pyramidal neurons (basal tree)	1,36	2.586	0.116	0.067	**11.326**	**0.002**	**0.239**	0.763	0.388	0.021
CA1 pyramidal neurons (apical tree)	1,38	2.242	0.143	0.056	**29.918**	**<0.0005**	**0.441**	**9.781**	**0.003**	**0.205**
CA1 pyramidal neurons (basal tree)	1,38	3.356	0.075	0.081	2.045	0.161	0.051	0.554	0.461	0.014
Sholl analysis - Hippocampus (Fig. 2f,j,n)										
Granular Neurons	1,35	3.482	0.070	0.090	**44.316**	**<0.0005**	**0.559**	**13.869**	**0.001**	**0.284**
CA3 pyramidal neurons (apical tree)	1,36	3.022	0.091	0.077	2.254	0.142	0.059	1.049	0.313	0.028
CA1 pyramidal neurons (apical tree)	1,38	0.059	0.809	0.002	3.546	0.067	0.085	2.456	0.125	0.061
Western Blot Data - Hippocampus (Fig.3a,b,d,e,f,g)										
LC3-II	1,30	**9.964**	**0.004**	**0.270**	1.112	0.299	0.040	0.270	0.608	0.010
P62	1,30	1.101	0.303	0.039	1.545	0.225	0.054	1.930	0.176	0.067
BDNF	1,30	0.526	0.475	0.019	0.128	0.724	0.005	2.126	0.156	0.073
PSD95	1,30	1.876	0.182	0.065	2.066	0.162	0.071	1.175	0.288	0.042
Synaptophysin	1,29	1.626	0.214	0.059	0.012	0.913	0.000	2.558	0.122	0.090
SNAP25	1,30	0.595	0.447	0.022	2.202	0.149	0.075	**4.944**	**0.035**	**0.155**

* p<0.05; ** p<0.01;*** p<0.001.

Supplementary material

Figure S1| Behavioral assessment of younger and older rats.

Table S2| List of correlations between the performance in working memory or behavioral flexibility tasks and the dendritic length of dorsal HPC neurons.

Figure S3| Cognitive performances in the WM, RM and BF tasks of the animals used in the morphological and molecular analysis.

Figure S4| Morphological analysis of neurons in the mPFC.

Table S5| Correlations between the performance in the behavioral flexibility task and the dendritic length of mPFC neurons.

Figure S6| Morphological alterations in the HPC and mPFC of individual young and old animals.

Table S7| List of correlations between dorsal HPC western blot data and the performance in the reference memory, working memory or behavioral flexibility tasks.

Figure S8| Dysregulation in autophagy signaling and dendritic pruning in the mPFC of older animals.

Table S9| Correlations between mPFC western blot data and the performance in the reference memory, working memory or behavioral flexibility tasks.

Figure S10| Full-length western blots for Figure 3c and supplementary Figure S8c.

Behavioral assessment
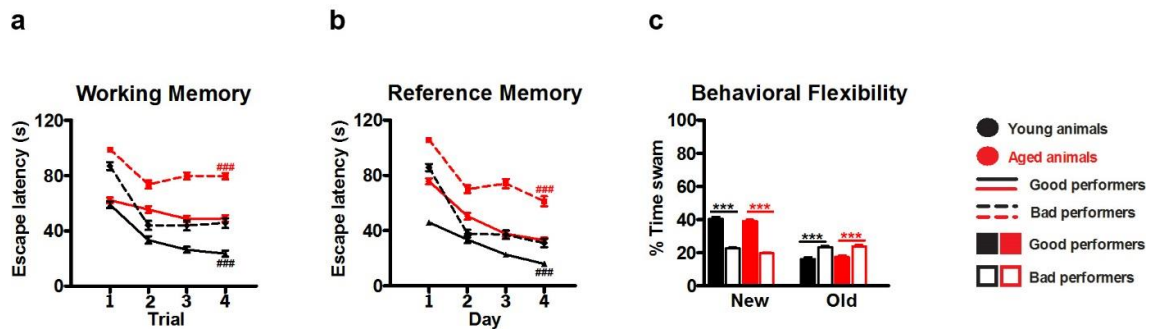
Figure S1| Behavioral assessment of younger and older rats. When similar age animals are clustered (see methods for details) in Good and Bad performers: **A, B)** Learning curves in the working **(A)** and reference memory task **(B)** of GPs and BPs for both younger and older rats. **C)** Results from the behavioral flexibility task. Average time spent on the four trials in each imaginary quadrant is given as a percentage of the total escape latency. Number of animals: working memory - older: GPs n=89, BPs n=87; younger: GPs n=63, BPs n=39; reference memory - older: GPs n=99, BPs n=77; younger: GPs n=61 BPs n=41; behavioral flexibility task - older: GPs n=62 BPs n=114; younger: GPs n=40 BPs n=61. Error bars represent SEM; * p<0.05; *** p<0.001.

Table S2| List of correlations between the performance in working memory or behavioral flexibility tasks and the dendritic length of dorsal HPC neurons.

			Old animals		Young animals	
			WM	BF	WM	BF
Granular neurons	**Dendritic length**	Pearson Correlation	**-0.458***	0.029	0.159	0.533
		Sig. (2-tailed)	**0.024**	0.894	0.572	0.050
		N	**24**	24	15	14
CA3 pyramidal neurons	**Apical dendritic length**	Pearson Correlation	**-0.431***	0.101	-0.230	**-0.649***
		Sig. (2-tailed)	**0.036**	0.645	0.410	**0.012**
		N	**24**	23	15	**14**
	Basal dendritic length	Pearson Correlation	-0.123	0.060	-0.173	**-0.585***
		Sig. (2-tailed)	0.567	0.785	0.538	**0.028**
		N	24	23	15	**14**
CA1 pyramidal neurons	**Apical dendritic length**	Pearson Correlation	**-0.418***	0.095	0.246	0.351
		Sig. (2-tailed)	**0.030**	0.644	0.377	0.219
		N	**27**	26	15	14
	Basal dendritic length	Pearson Correlation	**-0.436***	0.006	0.111	-0.095
		Sig. (2-tailed)	**0.023**	0.976	0.694	0.748
		N	**27**	26	15	14

*p<0.05
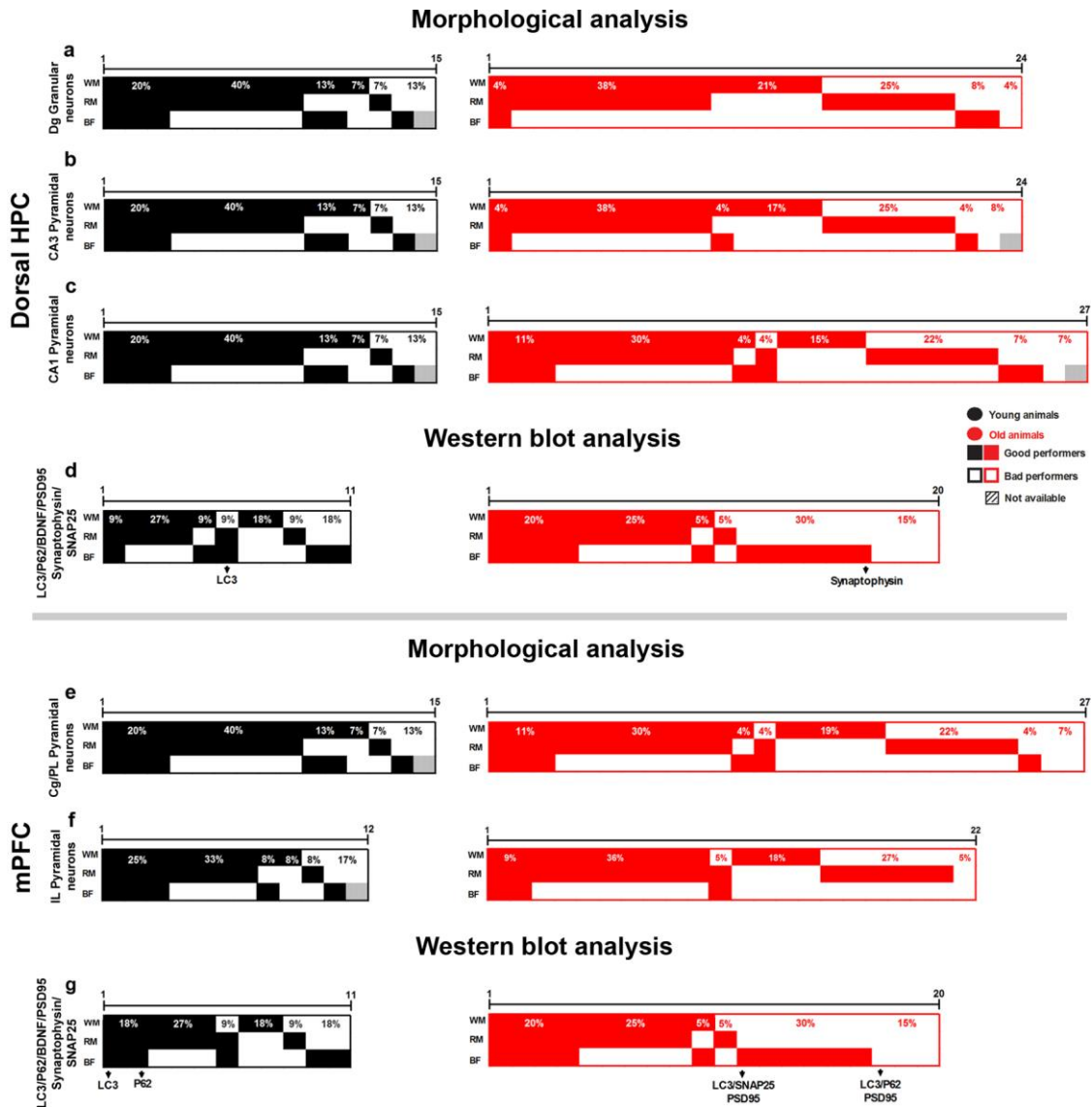
Figure S3| Cognitive performances in the WM, RM and BF tasks of the animals used in the morphological and molecular analysis. A, B, C, E and F) Represent the cognitive cluster of each animal (both young and aged) used for the analysis of the Dg, CA3, CA1, Cg/PL and IL, respectively. **D and G)** Represent the cognitive clusters of each animal (both young and aged) used for HPC **(A)** and mPFC **(B)** western blot analysis of LC3, p62, BDNF, PSD95, SNAP25 and Synaptophysin levels. Arrows indicate missing proteins in the analysis for each animal.

Morphological analysis - mPFC neurons
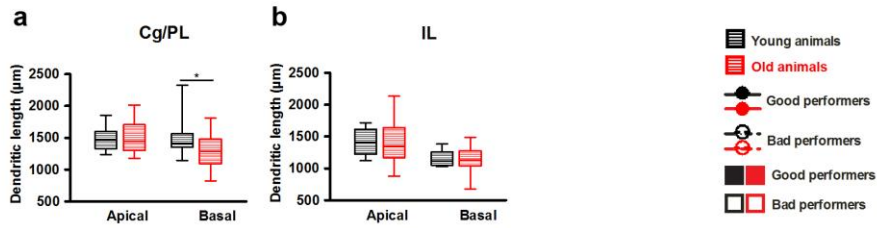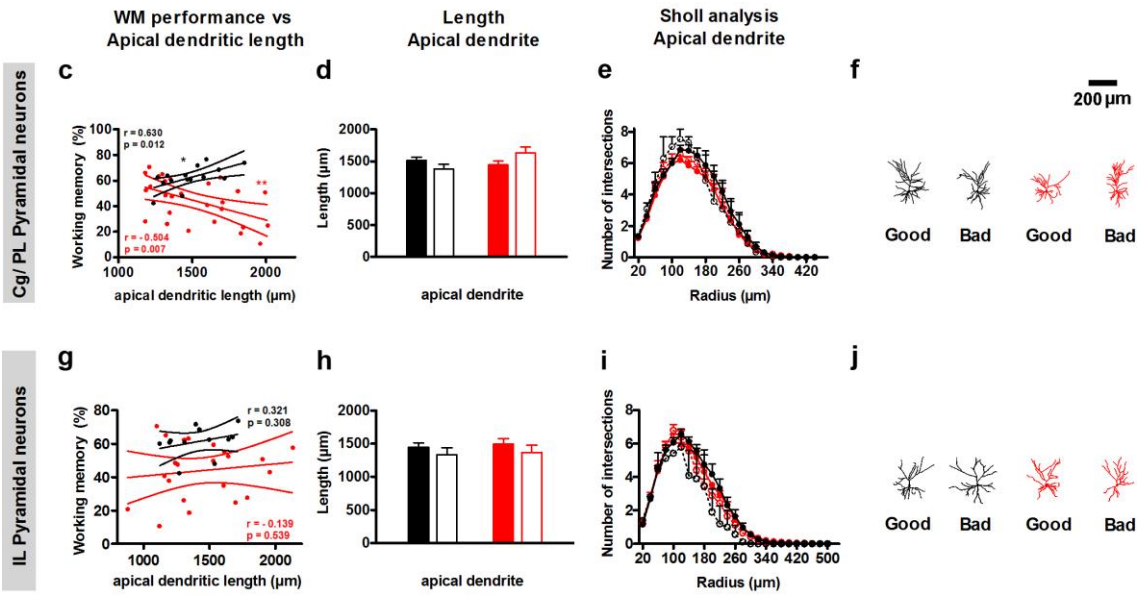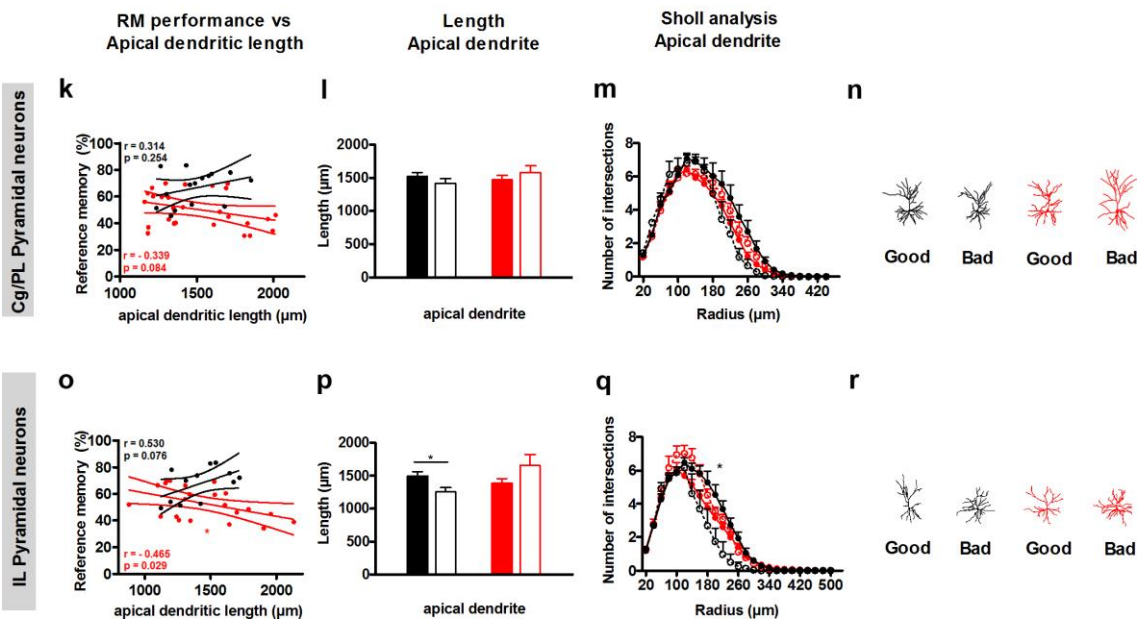
Figure S4| Morphological analysis of neurons in the mPFC. When a random sample of all animals is considered: **A** and **B)** Comparison of dendritic lengths of Cg/PL and IL pyramidal neurons of

the mPFC in both older (n=27 for Cg/PL and n=22 for IL neurons) and younger (n=15 for Cg/PL and n=12 for IL neurons) animals. **C** and **K)** Correlation between the individual performances of both younger and older rats in the working and reference memory tasks, respectively, and the apical dendritic length of Cg/PL pyramidal neurons. **G** and **O)** The same correlations were performed for the apical dendritic trees of IL pyramidal neurons. When similar age animals are clustered (see methods for details) in Good and Bad performers according to working or reference memory performance: **D** and **L)** Cg/PL pyramidal neuron apical dendritic lengths using, the performance in the working and reference memory tasks, respectively, to cluster the animals. **H** and **P)** The same procedure was performed for IL pyramidal neurons. **E** and **M)** Sholl analysis of the apical dendrites of Cg/PL pyramidal neurons. **I** and **Q)** The same analysis performed for IL pyramidal neurons. These graphs represent the mean number of intersections of apical dendrite branches with consecutive 20μm spaced concentric spheres. **F, J, N** and **K)** Reconstructions of representative Cg/PL and IL pyramidal neurons. Number of animals used for the analysis of the Cg/PL cortex: 15 younger animals (RM cluster GPs=10 and BPs=5; WM cluster GPs=12 and BPs=3) and 27 older animals (RM cluster GPs=18 and BPs=9; WM cluster GPs=17 and BPs=10). For the IL cortex the number of animals used was: 12 younger animals (RM cluster GPs=8 and BPs=4; WM cluster GPs=9 and BPs=3) and 22 older animals (RM cluster GPs=17 and BPs=5; WM cluster GPs=14 and BPs=8). Error bars represent SEM, doted lines represent confidence intervals and continuous lines are linear fits; $*p<0.05$. (WM – working memory; RM – reference memory).

Table S5 | Correlations between the performance in the behavioral flexibility task and the dendritic length of mPFC neurons.

			Old animals	Young animals
			BF	BF
Cg/PL neurons	**Apical dendritic length**	Pearson Correlation	-0.042	0.491
		Sig. (2-tailed)	0.836	0.075
		N	27	14
	Basal dendritic length	Pearson Correlation	-0.128	-0.422
		Sig. (2-tailed)	0.524	0.133
		N	27	14
IL neurons	**Apical dendritic length**	Pearson Correlation	-0.208	**0.611**
		Sig. (2-tailed)	0.340	**0.046**
		N	22	**11**
	Basal dendritic length	Pearson Correlation	-0.091	-0.276
		Sig. (2-tailed)	0.679	0.411
		N	22	11

*p<0.05

Figure S6| Morphological alterations in the HPC and mPFC of individual young and old animals. A and B) Representation of all the morphological alterations simultaneously occurring in the HPC (Dg granular, CA3 and CA1 pyramidal neurons) and mPFC (Cg/PL and IL pyramidal neurons), regions of both young **(A)** and old group **(B).** Asterisk represents missing analysis. The color scale is relative to the minimum and maximum length within each individual region.

Table S7| List of correlations between dorsal HPC western blot data and the performance in the reference memory, working memory or behavioral flexibility tasks.

				Old animals			Young animals		
				RM	WM	BF	RM	WM	BF
HIPPOCAMPUS	Autophagy	LC3-II	Pearson Correlation	**0.523·**	0.288	0.173	**0.790··**	0.042	-0.151
			Sig. (2-tailed)	**0.018**	0.218	0.467	**0.004**	0.904	0.677
			N	**20**	20	20	**11**	11	11
		p62	Pearson Correlation	**-0.489·**	-0.345	-0.092	-0.012	0.207	-0.291
			Sig. (2-tailed)	**0.029**	0.136	0.699	0.972	0.541	0.415
			N	**20**	20	20	11	11	11
	Dendritic growth	BDNF	Pearson Correlation	**-0.577··**	**-0.447·**	0.183	0.382	0.610	-0.289
			Sig. (2-tailed)	**0.008**	**0.048**	0.441	0.247	0.061	0.417
			N	**20**	**20**	20	11	11	11
	Synaptic marker	PSD95	Pearson Correlation	**-0.448·**	**-0.513·**	0.148	0.184	-0.084	-0.058
			Sig. (2-tailed)	**0.048**	**0.021**	0.533	0.588	0.807	0.874
			N	**20**	**20**	20	11	11	10
		Synaptophysin	Pearson Correlation	-0.453	-0.406	0.290	0.286	-0.005	-0.165
			Sig. (2-tailed)	0.052	0.085	0.228	0.394	0.988	0.649
			N	19	19	19	11	11	11
		SNAP25	Pearson Correlation	**-0.560·**	-0.339	-0.111	0.208	0.039	-0.342
			Sig. (2-tailed)	**0.010**	0.143	0.641	0.539	0.909	0.334
			N	**20**	20	20	11	11	11

*p<0.05; **p<0.01

Western blot analysis - mPFC
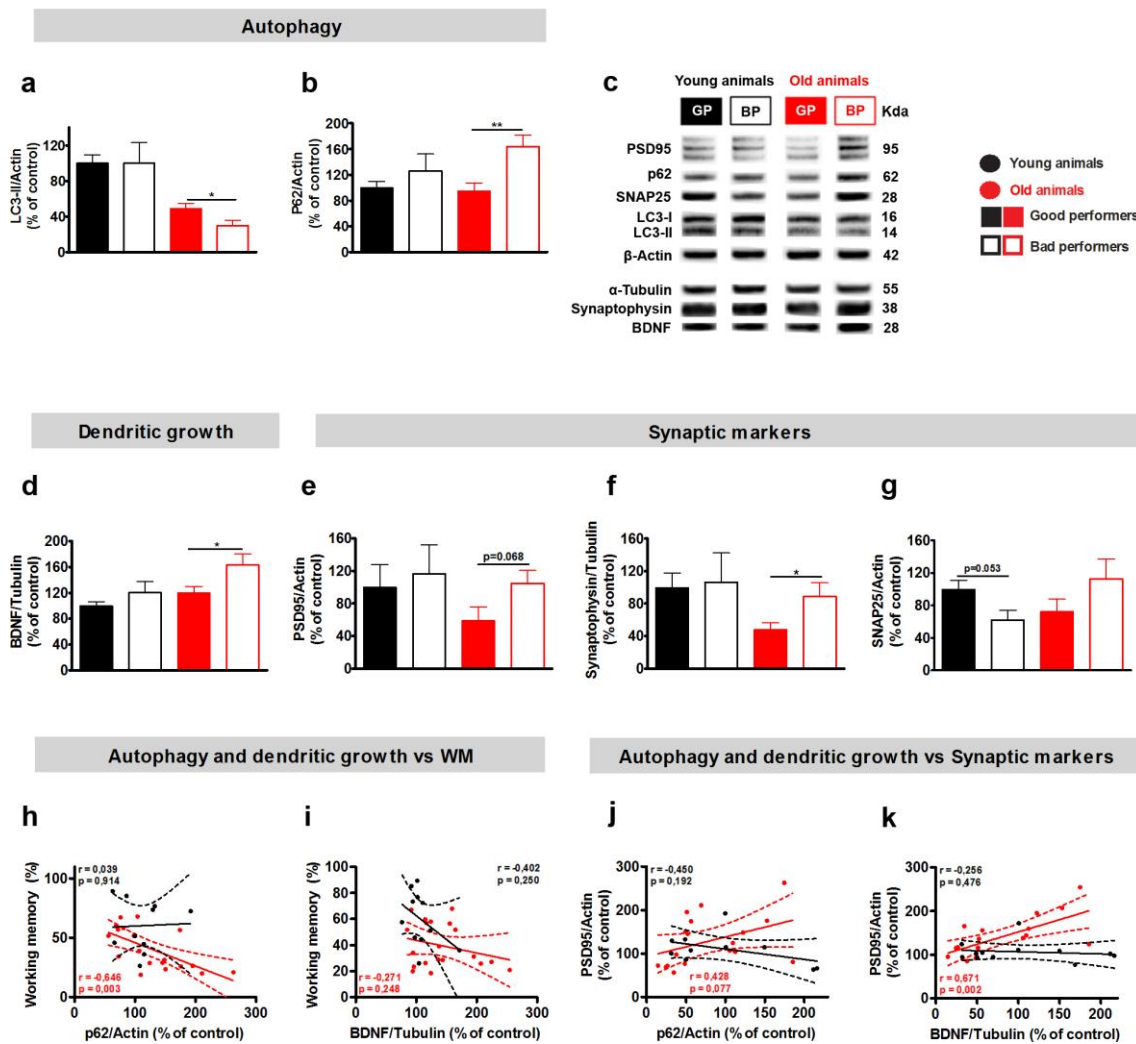
Figure S8| Dysregulation in autophagy signaling and dendritic pruning in the mPFC of older animals. Working memory performance was used to cluster both younger and older animals in GPs and BPs. **A** and **B)** Levels of autophagy markers, LC3-II **(A)** and p62 **(B)**, normalized to actin. **D)** BDNF levels normalized to tubulin. **E, F, G)** Levels of synaptic markers PSD95, SYP and SNAP25 normalized to actin, tubulin, and actin respectively. **C)** Representative western blots of PSD95, p62, SNAP25, LC3, Actin, Tubulin, SYP, and BDNF. For each protein, the blots were cropped from different parts of the same gel. **H** and **I)** Correlation between working memory performance and p62 or BDNF levels, respectively. **J** and **K)** Correlation between PSD95 and p62 or BDNF levels, indicating an association between synaptic marker levels, autophagy and dendritic growth. Number of animals: 10-11 younger animals (GPs=6-7 and BPs=4) and 18-20 older animals (GPs=10 and BPs=8-10). Error bars represent SEM, doted lines represent confidence intervals and continuous lines are linear fits; $*p<0.05$; $**p<0.01$; $***p<0.001$.

Table S9 | Correlations between mPFC western blot data and the performance in the reference memory, working memory or behavioral flexibility tasks.

mPFC				Old animals			Young animals		
				RM	WM	BF	RM	WM	BF
	Autophagy	LC3-II	Pearson Correlation	0.387	0.445	0.003	-0.462	-0.093	0.016
			Sig. (2-tailed)	0.112	0.064	0.989	0.179	0.798	0.968
			N	18	18	18	10	10	10
		p62	Pearson Correlation	**-0.504·**	**-0.646··**	-0.003	-0.573	0.039	-0.444
			Sig. (2-tailed)	**0.028**	**0.003**	0.991	0.083	0.914	0.231
			N	**19**	**19**	19	10	10	10
	Dendritic growth	BDNF	Pearson Correlation	-0.150	-0.271	-0.203	-0.218	-0.425	-0.099
			Sig. (2-tailed)	0.527	0.248	0.390	0.520	0.193	0.786
			N	20	20	20	11	11	11
	Synaptic markers	PSD95	Pearson Correlation	-0.113	-0.217	0.010	0.158	-0.020	**0.702·**
			Sig. (2-tailed)	0.654	0.386	0.968	0.643	0.954	**0.024**
			N	18	18	18	11	11	**10**
		Synaptophysin	Pearson Correlation	-0.199	-0.304	-0.330	-0.262	0.297	0.055
			Sig. (2-tailed)	0.401	0.192	0.155	0.436	0.376	0.879
			N	20	20	20	11	11	11
		SNAP25	Pearson Correlation	-0.191	-0.131	0.096	**0.755··**	0.449	0.142
			Sig. (2-tailed)	0.434	0.593	0.697	**0.007**	0.166	0.696
			N	19	19	19	**11**	11	11

·p<0.05; ··p<0.01
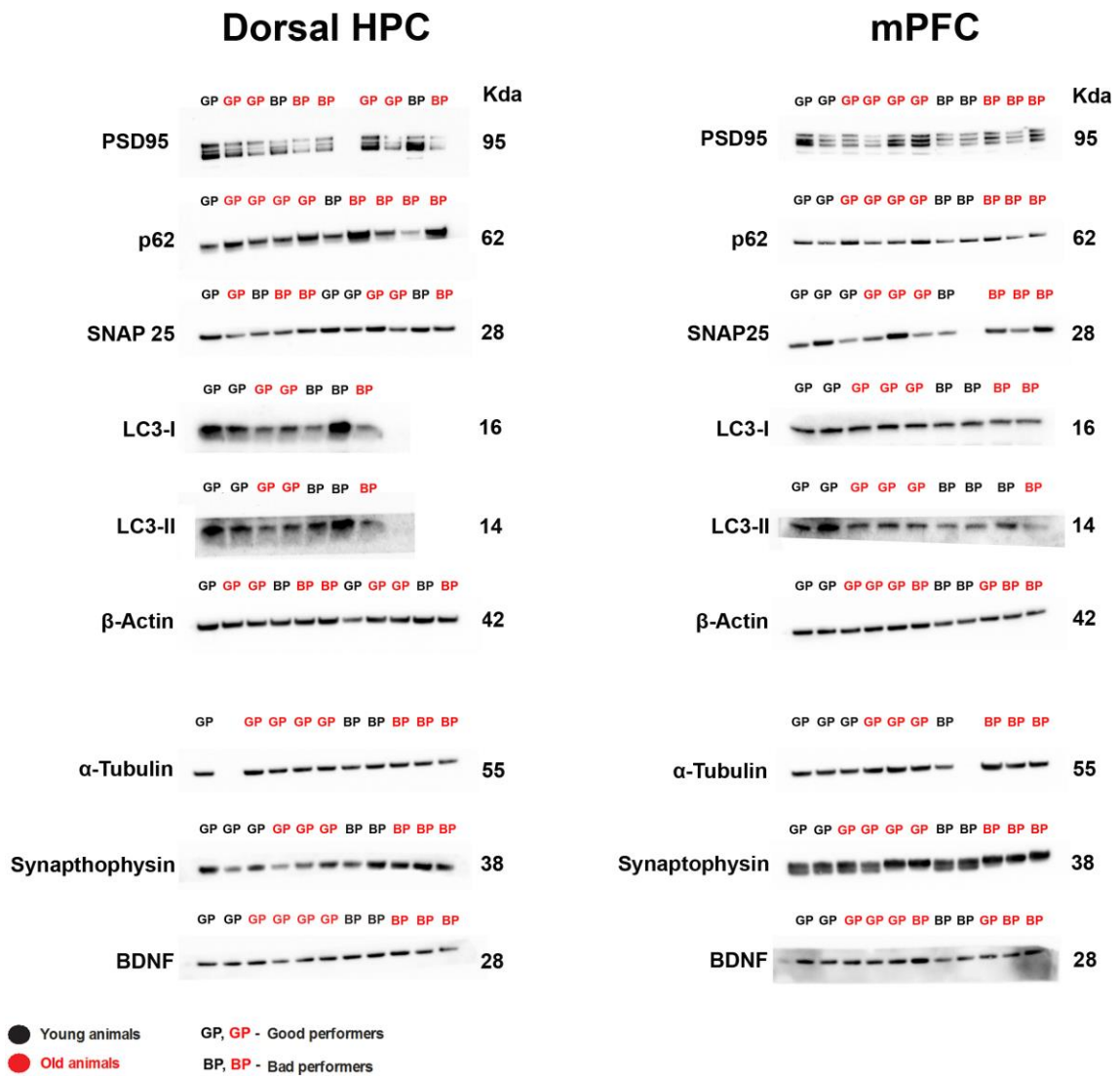
Figure S10| Full length western blots. Full-length western blots for figure 3c and supplementary figure S8c.

Bigger is worse: volumetric correlates of age-related changes in hippocampus and medial prefrontal cortex in the rat

Mota C, Pereira das Neves S, Sousa N, Sousa JC & Cerqueira JJ

Bigger is worse: volumetric correlates of age-related changes in hippocampus and medial prefrontal cortex in the rat

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1. Abstract

The world population is increasingly old, making aging research a top priority. Although human aging is generally associated with decreased cognition, several individuals retain their abilities until late in life. Understanding why this happens can help the promotion of "healthy aging". We have previously shown that older rats possess poorer spatial learning and behavioral flexibility than younger subjects, but the degree of cognitive decline was highly variable, with some old subjects performing as well as, or even better than their younger counterparts. Given this variability, we clustered younger and older animals according to individual cognitive performance as good and bad performers. Using a battery of water maze tasks, and a detailed stereological analysis, these behavioral differences were now correlated with volumetric alterations in the hippocampus (HPC) and medial prefrontal cortex (mPFC). While in younger animals, better cognitive performance was associated with increased HPC and mPFC volumes, in the older group, better performance was associated with lower volume on both brain structures. Interestingly, while working memory performance was only related with alterations in the mPFC, the reference memory performance correlated with both hippocampal and mPFC volumes.

These results, together with our previous findings relating longer dendritic trees and autophagy deficits to cognitive deficits in the older population, suggest that volumetric alterations are associated with age-related HPC and mPFC-dependent behavior deficits. Therefore, the current work once more supports the notion that while for the younger bigger is better, for the older, smaller is definitively the best.

2. Introduction

Normal aging is a process which inevitably triggers plastic and adaptive changes in the brain, which generally translate as a decline in cognitive abilities (Erickson & Barnes, 2003). However, one of the most striking characteristics of human aging is its heterogeneity (Ardila, 2007; Santos *et al.*, 2013). Understanding why some individuals' cognition seems to be unaffected by age could promote the design of strategies to prevent this age-related deleterious declining.

Using rats as models of cognitive aging, we recently showed that, as in humans, the profile of cognitive deficits in rodents is broad and the severity of the cognitive decline associated with aging is highly variable (Mota *et al.*, 2018). Furthermore, we previously showed that alterations in the dendritic length of neurons from hippocampus (HPC) and medial prefrontal cortex (mPFC) underpin the heterogeneity observed in the performance of younger and older animals, with a twist. Indeed, it seems that while for younger animals "bigger is better" for older animals "smaller is definitely better". Moreover, we provided evidence

86

that, in older animals, dendritic length differences, and concomitant behavioral heterogeneity, can be ascribed to variations in neurotrophin levels and, more importantly, autophagic activity, leading to dendritic pruning deficits (Mota *et al.*, 2018).

Several studies have also shown that volume alterations in the HPC and mPFC areas could be an important determinant for cognitive impairment. As it was previously reported that neuronal dendritic remodelling is directly related with volumetric alterations (Cerqueira *et al.*, 2005, 2007), in the present work, we hypothesized that our previous findings (Mota *et al.*, 2018) should translate in equivalent volumetric changes in HPC and mPFC areas.

Historically, it has been presumed that brain volume loss in HPC and mPFC areas underpin age-related cognitive decline, in both humans (Jernigan *et al.*, 1991; Golomb *et al.*, 1993 Driscoll *et al.*, 2003, 2009; Freeman *et al.*, 2008; Raz *et al.*, 2010) and rats (Rapp *et al.*, 1999; Driscoll *et al.*, 2006; Yates *et al.*, 2008). However, this view has been recently brought into question. For example, in humans, some studies reported no volumetric changes in the HPC during normal aging (Sullivan *et al.*, 1995; Raz, 1996) and another study suggested that larger HPC are associated with less effective memory performance in healthy young adults (Molnár & Kéri, 2014). Important to highlight is that most of these studies failed to accommodate the evidence of individual heterogeneity in aging.

Despite reports of age-related structural variations, little is known regarding the relationship between these variations and age-related decline in HPC and mPFC-dependent learning and memory. Therefore, in an extension of our previous work, the present study intends to fill this gap in the structure-function relationship by the parallel evaluation of cognitive performance and volume alterations in HPC and mPFC. Briefly, after cognitive characterization of both younger and older animals as good and bad performers (GPs, BPs), a detailed stereological analysis was applied to estimate the volumes of the main divisions of the HPC formation and mPFC. This data showed, once again, that bigger HPC and mPFC volumes correlate to worse cognitive performance in the older rodent population.

3. Methods

3.1. Animals

All procedures were carried out in accordance with local regulations (Decreto-Lei n.º 113/2013) and European Union Directive 2010/63/EU on animal care and experimentation. Animal facilities and the people directly involved in animal experiments were certified by the Portuguese regulatory entity – DGAV (Direção-Geral de Alimentação e Veterinária). All protocols were approved by the Ethics Committee of the

Life and Health Sciences Research Institute (ICVS). All the male Wistar Han rats (Charles River Laboratories, Barcelona, Spain) used in the study were housed in groups of 2 and maintained under standard laboratory conditions: artificial 12h light/dark cycle (lights on from 08:00 a.m. to 08:00 p.m.); room temperature 22°C; *ad libitum* access to food and water. From a total of 176 old (22-24-month-old) and 102 younger (4-6-month-old) male rats, previously behaviorally characterized by Mota *et al.*, 2018 in a battery of water maze-based tests, a subset of 13 younger and 32 older animals were used in this study. Animals were clustered, within the original animal group, into GPs and BPs, according to their performance in each water maze-based test (Mota *et al.*, 2018), and subjected to stereological analysis. The remaining animals were sacrificed at different time points for several other analyses not included in the present study. All behavioral testing was conducted during the daytime.

3.2. Behavioral assessment

The cognitive status of all the animals was assessed based on their performance in a series of tasks using the water maze (working and reference memory, behavioral flexibility). For more details see Mota *et al.*, 2018.

3.3. Histological procedures

Two months after the behavioral evaluation, 32 older rats and 13 younger rats were randomly selected, deeply anesthetized with sodium pentobarbital and perfused transcardially with 4% paraformaldehyde solution for glycolmethacrylate inclusion. Whenever the histological conditions affected the final quality of the ample sections, these were excluded from de analysis therefore reducing the number of available animals for the study of some brain regions.

Glycolmethacrylate inclusion

Brains were removed and placed in fixative. After 4 weeks, brains were split into two hemispheres by a midsagittal section and processed for stereology, according to the procedure described previously by Keuker *et al.*, 2001. Briefly, they were included in glycolmethacrylate (Tecnovit 7100; Heraeus Kulzer, Werheim, Germany) and every other microtome-cut section (30μm) was then collected on a gelatinized slide, stained with Giemsa, and mounted with Entellan New (Merck, Darmstadt, Germany).

Structural analysis

In order to ensure an unbiased analysis, slides were re-coded by the lab technician (not otherwise involved in the research) as soon as they were prepared and all stereological analyses were done blind to animal age or performance group. To minimize bias, codes were only broken after all data was collected and entered into the database.

Region and layer boundaries

We analyzed three areas of the mPFC: cingulate (Cg), prelimbic (PL) and infralimbic (IL) cortices. Each mPFC subregion was further divided parallel to the surface in three easily distinguishable layers (layer I, layer II and layers III-VI), based on cell packing. The third level was considered as a whole because a clear boundary between its layers could not be found in the mPFC (for more details see Cerqueira *et al.*, 2005). The HPC was divided in dorsal (DHPC) and ventral counterpart (VHPC) according to Pinto *et al.*, 2015, and was analyzed according to its main anatomical divisions: dentate gyrus (DG) (including hilus, granule cell layer, and molecular layer), cornu ammonis regions 3 and 1 (CA3 and CA1) (including strata oriens, pyramidale and radiatum). The above-mentioned regions were outlined according to the atlas of Paxinos & Watson, 1998, based on noticeable cytoarchitectural differences (Palomero-Gallagher & Zilles, 2004; Vogt *et al.*, 2004).

Stereological procedures

Volume estimations were performed using StereoInvestigator software (MicroBrightField, Williston, VT) and a camera (DXC390; Sony, Tokyo, Japan) attached to a motorized microscope (Axioplan 2; Zeiss, Oberkochen, Germany). Cavalieri's principle (Gundersen *et al.*, 1988) was used to assess the volume of each region. Briefly, after starting at a random position, every 8th (for IL, PL and anterior Cg cortex), 20th (for DHPC) and 10th (for VHPC) section was used and its cross-sectional area was estimated by point counting (final magnification x112). For this, we randomly superimposed onto each area a test-point grid in which the interpoint distance, at tissue level, was 75 μm for IL layers I and II; 100 μm for IL layer III-VI and PL level I and II;150 μm for PL layer III-VI, Cg layer I and II and the three layers of the DG; 250 μm for Cg layer III-VI and the three layers of CA3 and CA1. The volume of the region of interest was determined from the number of points that fell within its boundaries and the distance between the systematically sampled sections. The estimation of volumes of the different regions of the HPC and mPFC formation were undertaken in the right hemisphere.

3.4. Statistical analysis

All statistical analysis was conducted in the SPSS software package version 24 (IBM corporation, Armonk, New York). After confirmation of normality and homogeneity, appropriate statistical tests were applied to the data. To facilitate direct comparisons between different tests, results of all behavioral tests were converted to a 0-100% scale, where 0% indicates worst possible performance (120s to reach the platform for the working and reference memory tests or no time in target quadrant for the behavioral flexibility task) and 100% indicates best possible performance (0s to reach the platform for the working and reference memory tests or 120s in target quadrant for the behavioral flexibility task). Also, in order to allow individual correlations between the performance of the animals in the working and reference memory test, which consist of several testing days, and structural parameters, we calculated a performance index for each test by employing the following formula: Performance index=(P1+(P3+P4)/2)/2 where Pn represents the average performance of each animal on trial n (for working memory) or day n (for reference memory); importantly, the index value can be directly read as the average performance of each animal per trial/day. Regarding the behavioral flexibility test, the percentage of time spent in the target quadrant (performance index) was used to assess the individual correlations. Clustering of animals in GPs and BPs was done using the k-means cluster analysis (for more details see Mota *et al.*, 2018). Comparisons between groups (younger versus older animals and good versus bad performers) were done using the two-tailed *t*-test (for values of a single test) or the repeated-measures ANOVA (for repeated testing). Two-way ANOVA was used to evaluate the impact of age and the effect of group performance in further structural data. Pearson product-moment correlations were computed between continuous variables. Measures of effect size (Cohen's *d*, partial Eta-squared - or Pearson correlations - r) are presented whenever appropriate. Differences were considered to be significant if *p<0.5; **p<0.01; ***p<0.001.

4. Results
4.1. Behavioral data

A k-means clustering was performed to classify younger and older animals according to their performance in working memory, reference memory or behavioral flexibility tasks. This resulted, for each test and age, in two groups of subjects (GPs and BPs) (for more details see Mota *et al.*, 2018).
In figure 1a,b, and c the behavioral performance and group membership of the subset of the animals used for the stereological analysis is presented. A two-way ANOVA analyses of the learning curves for the

working and reference memory tasks (Fig. 1a,b) revealed a significant effect of age and performance in both tasks, but not an interaction between these two factors (Table 1). Regarding the behavioral flexibility test, only the time spent in the new quadrant presented a significant effect of performance, but not of age, with a significant interaction between them (Table 1). Within group comparisons showed, that for older and younger individuals, significantly different performances were observed between GPs and BPs for all the three cognitive tasks evaluated (Table 1 and Fig. 1a,b,c). Overall, in memory tests, both for older and younger adult rats, the GPs performed better than BPs (Fig. 1a,b,c).

Behavioral assessment
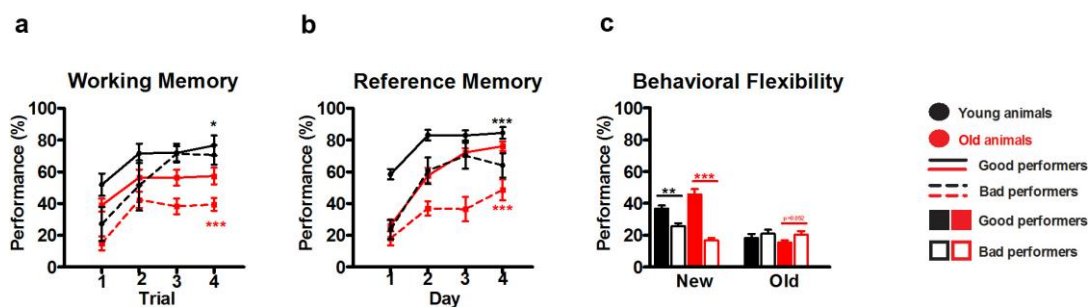
Figure 1| Performance clustering of younger and older rats according to working memory, reference memory and behavioral flexibility water maze tests. A) Learning curve in the working memory (older: GPs n=15, BPs n=17; younger: GPs n=10, BPs n=4) and **B)** reference memory tasks (older: GPs n=18, BPs n=14; younger: GPs n=9, BPs n=5). **C)** Results from the behavioral flexibility task (older: GPs n=8, BPs n=24; younger: GPs n=7, BPs n=5). Error bars represent SEM; * p<0.05; ** p<0.01; *** p<0.001.

4.2.   Enlargement of the hippocampal and mPFC volume with age

Older animals displayed an enlargement of the volume of the HPC and mPFC when compared to their youngster counterparts (Table 2 and Fig. 2a and 3b, respectively). As there is strong evidence supporting a functional dissociation between the DHPC and the VHPC: the DHPC being predominantly dedicated to cognitive processing whereas its ventral counterpart more implicated in emotional processing, our analysis also focused on the dorsal and ventral subdivisions of the HPC. In agreement with the data of the whole HPC volume, the volume of the DHPC was significantly increased with age (Table 2 and Fig. 2a). Interestingly, no differences were found in the volume of the VHPC between older and younger animals (Table 2 and Fig. 2a). Therefore, we next analyzed in more detail the various subregions of the

dorsal hippocampal formation, namely: the DG (molecular, granular layer and hilus), the CA1 and the CA3 (stratra oriens, pyramidale and radiatum). In general, for each of these subdivisions, and in accordance to what was observed in the total volumetric analysis, older animals presented an enlargement in most of these structures (Table 2 and Fig. 2b). These alterations were observed at the level of the molecular layer and hilus of DG, stratum radiatum of CA3 and stratum oriens and radiatum of CA1 (Table 2 and Fig. 2b). Similar to this approach, we also dissected the mPFC formation on its subregions: Cg, PL and IL areas; and for each of these areas, we analyzed three different layers: layer I, layer II and layer III-VI. Our assessments showed that older animals presented higher volumes of the PL and IL areas when compared to younger animals, but no changes in Cg volume (Table 2 and Fig. 3a). We then checked the individual layers of these subregions and observed an increase in the volume of all the layers of the older PL (Table 2 and Fig. 3b). Regarding the IL, only differences in layer I were found. Once again, older animals presenting an increased volume when compared to the younger counterparts (Table 2 and Fig. 3b). The Cg showed no age-related alterations in volume (Table 2 and Fig. 3b).

4.3. Differential alterations in the volume of the hippocampus are correlated with the individual performance, of both younger and older animals, in the reference memory task

Volumetric analysis of the HPC of both younger and older animals revealed that alterations in the volume of the hippocampal formation correlated with the individual performance in the reference memory task (Table 3 and Fig. 2e), but not in the other cognitive tasks (working memory - younger animals: $r^2 = 0.209$, $p = 0.493$; older animals: $r^2 = -0.185$, $p = 0.366$; behavioral flexibility - younger animals: $r^2 = 0.460$, $p = 0.155$; older animals: $r^2 = 0.082$, $p = 0.689$). Interestingly, while for younger animals, a good performance in the behavioral task was correlated with a higher HPC volume, in old animals this correlation was reversed, with better performers having smaller HPC than worse ones (Table 3 and Fig. 2e). In addition to the whole HPC volume, we also analyzed the dorsal and ventral subdivisions. Only the volume of the DHPC, but not the VHPC, was significantly correlated with reference memory performance, with younger and older animals showing, again, correlations in opposite directions (Table 3 and Fig. 2f,g). Detailing the volumetric correlates by further subdividing the DHPC revealed that, in older subjects, cognitive performance was significantly inversely correlated with dorsal DG and dorsal CA1 subdivisions but not any dorsal CA3 subdivisions (Table 3). In contrast, in younger animals, only the volumes of the dorsal DG granular layer and dorsal CA1 stratum radiatum were significantly correlated with reference memory performance (Table 3).

Volumetric analysis - Hippocampus
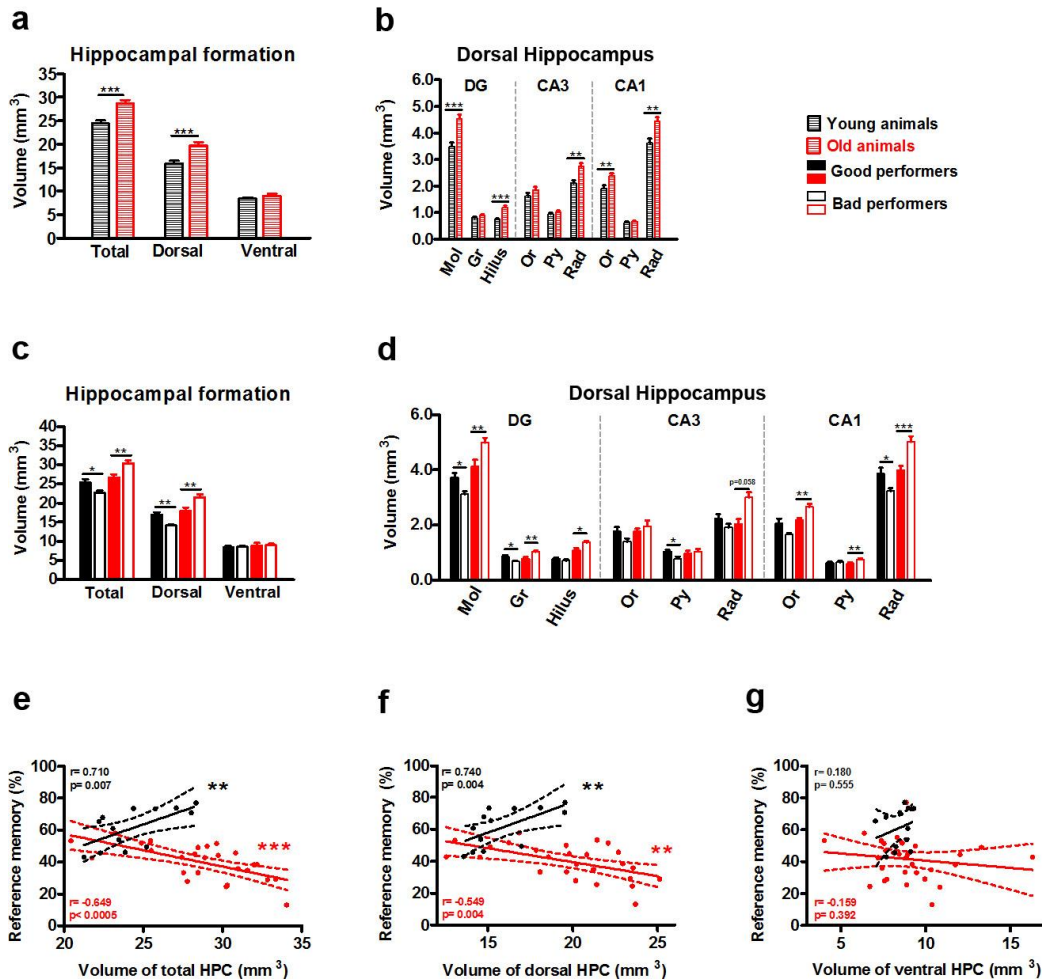
Figure 2| Structural alterations in the hippocampal formation correlate with the performance of both younger and older rats in the reference memory task. A) Total, dorsal and ventral volume of the hippocampal formation. Older animals present a volumetric increase of the hippocampal formation due to an increase in the volume of the dorsal hippocampus. **B)** Analysis of the volumes of different subregions of the dorsal hippocampus shows a systematic increase in older animals. **C)** Mean volumes of the hippocampal formation for younger and older animals, both GPs and BPs. **D)** Mean volumes of the different subregions of the dorsal hippocampus for younger and older animals, both GPs and BPs. Note that for older BPs the areas that contribute to the volume increase are mainly the DG and CA1. **E, F** and **G)** Correlation of the individual performances of both younger and older rats in the reference memory task with: **(E)** total volume of hippocampal formation **(F)** dorsal and **(G)** ventral hippocampus. Number of animals used in volumetric assessments was: 13 younger animals GP=8 and BP=5) and 31 older animals (GP=18-14 and BP=13-12). Continuous lines in **E, F** and **G** are Gaussian fits. Error bars represent SEM; $*p < 0.05$; $** p < 0.01$; $***p < 0.001$.

Additionally to individual correlations, we also compared the hippocampal volumes of GPs and BPs in each age group. Using the reference memory performance to cluster the animals, two-way ANOVA analysis reported a significant effect of age, but not of performance group, in total and DHPC volumes, with a significant interaction between both (Table 4 and Fig. 2c). As expected, analysis of the ventral pole did not reveal any effect of age, performance group or any interaction between them (Table 4 and Fig. 2c). Further analysis of the subdivisions of the DHPC, revealed also a significant effect of age, but not of performance group, in the volume of the molecular layer of DG, stratum oriens and radiatum of CA1 regions, with a significant interaction between both (Table 4 and Fig. 2d). Regarding the granular layer, only a significant interaction between age and performance was reported (Table 4 and Fig. 2d). A significant effect of age was observed for the stratum radiatum of CA3 and for hilus, without an effect of performance group, neither an interaction between them (Table 4 and Fig. 2d). Within group comparisons further revealed that, in younger animals, the volume of total and DHPC were significantly higher in GPs than in BPs, while the volume of the ventral region was similar between the two groups (Table 5 and Fig. 2c). A subsequent analysis of the different subregions of the DHPC revealed that the volume of the molecular and granular layers of the DG, as well as the volume of the pyramidal layer of CA3 and the stratum radiatum of CA1, were contributing to these differences (Table 5 and Fig.2d). In older animals, we observed an opposite effect. The volume of total and DHPC were significantly higher in BPs than in GPs, while the volume of the ventral region was also similar between the two groups (Table 5 and Fig.2c). Here, only the volumes of all the layers of the DG and CA1 subregions contribute significantly to these differences (Table 5 and Fig. 2d).

4.4. Volumetric alterations in mPFC correlate with individual performance in both working and reference memory task

Contrary to the HPC, the behavioral correlates of mPFC volume were different for younger and older animals. While in the former age group, regional volume was only positively correlated with working memory performance, in the latter the only significant, and negative correlation was with the reference memory task (Table 6 and Fig. 3e,k). Concerning the behavioral flexibility task, no correlations were found (younger animals: r^2 = 0.605, p = 0.084; older animals: r^2 = -0.119, p = 0.617). The total volume of the mPFC structure measured in this study comprises the volume of Cg, PL and IL areas. Performance of a similar analysis for the different areas reveals that, for older animals, the negative correlation between reference memory and mPFC volume is exclusively driven by Cg (Table 6 and Fig. f,g,h), while for younger

animals, the positive correlation described above, between working memory performance and mPFC volume is driven by alterations in PL and IL, but not Cg (Table 3 and Fig. 3l,m,n). Of note, IL volume only correlates with the reference memory performance in younger subjects (Table 6 and Fig. 3h).

In addition to individual correlations, we also compared average mPFC volumes between the different performance groups. Regarding group clustering based on reference memory performance, two-way ANOVA analysis reported a significant effect of age, but not of performance group, in the volume of: total mPFC, PL and IL, but not Cg , while the interaction between the two factors was only significantly different in the latter (Table 4 and Fig. 3c). When animals where classified by working memory performance, two-way ANOVA analysis similarly revealed a significant effect of age, but not a of performance group, in the alterations observed in the volume of total mPFC, PL and IL, but not Cg, while the interaction between these two factors was only significantly different in the volume of total mPFC and PL (Table 4 and Fig. 3i). In addition, within group comparisons using the reference memory performance to cluster the animals, showed that, in older animals, the volume of the Cg was significantly higher in BPs than in GPs, and these differences were maintained along all the Cg layers (Table 5 and Fig. 3c,d). Contrary, in younger animals, a higher volume of layer I of Cg and layers III-VI of IL was observed when comparing GPs to BPs (Table 5 and Fig.3c,d). When the working memory task was used to cluster the animals, differences regarding the total volume of mPFC, PL and IL were observed only for younger animals (Table 5 and Fig. 3i). Also, significant volumetric alterations were reported for all the layers of PL area (Table 5 and Fig. 3j). Once more, these differences are related with a higher volume in GPs than in BPs. Concerning the older group, and in opposition to the previous data, only a significant increase in the volume of layer I of PL was observed in the BPs when compared with de GPs (Table 5 and Fig. 3j).

Volumetric analysis - mPFC
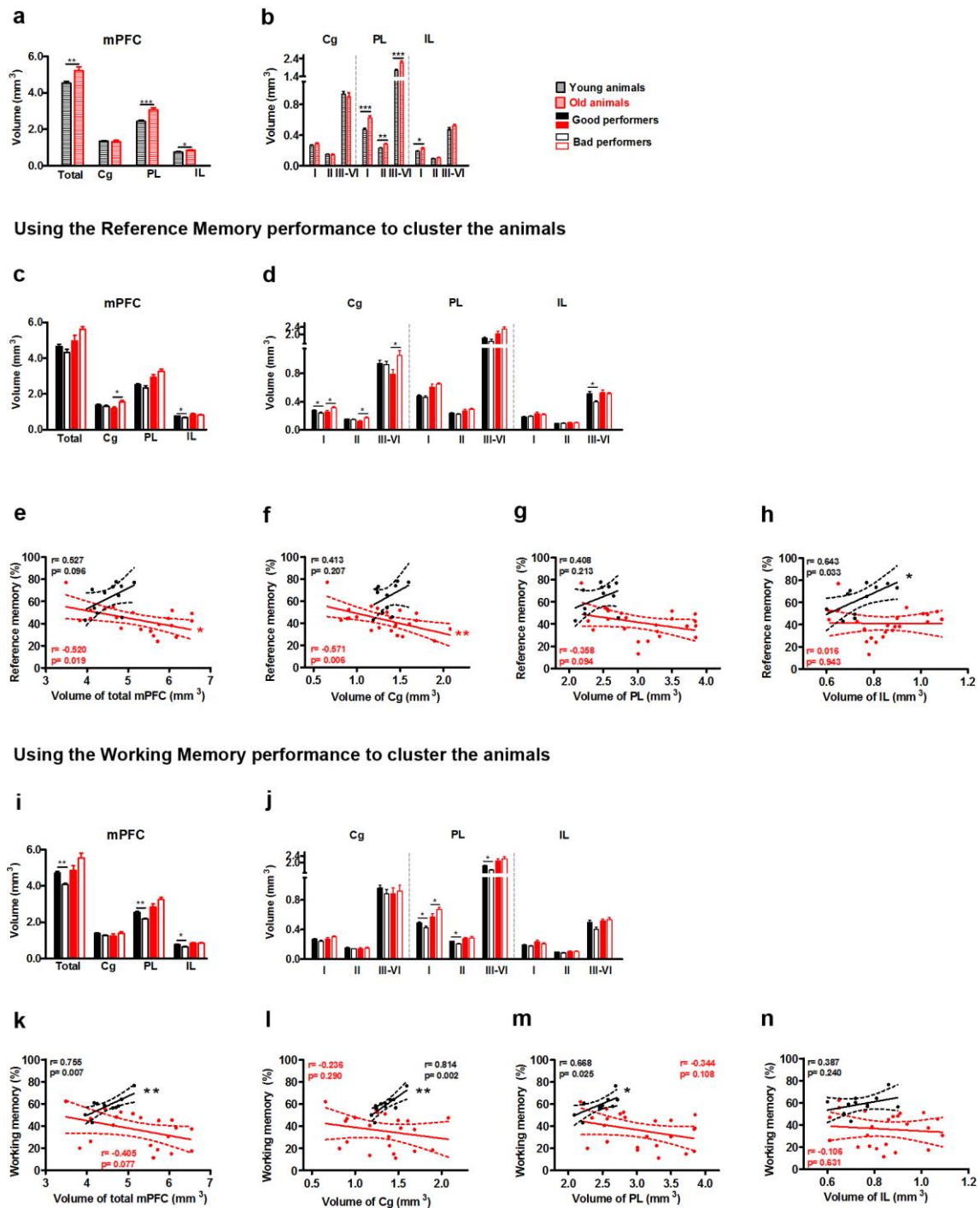
Figure 3| Volumetric alterations in the mPFC correlate with the performance of younger and older rats in the reference and working memory tasks. A) Volumetric assessment of the mPFC formation. Older animals present a volumetric increase of the mPFC due to an increase in the volume of the PL and IL cortices. **B)** Volume of the three different layers (layer I, II or III-VI) of Cg, PL and IL area. **C, D, I** and **J)** Mean volumes of the mPFC and of its subdivisions for both younger and older animals, classified as GPs and BPs based on the reference memory performance **(C,D)** or working

memory performance **(I,J)**. **E-H)** Correlation of the individual performances of both younger and older rats in the reference memory task with: **(E)** total volume of mPFC, **(F)** volume of Cg, **(G)** PL and **(H)** IL cortices. **K-N)** Correlation of the individual performance of both younger and older rats in working memory task with: **(K)** total volume of mPFC, **(L)** volume of Cg, **(M)** PL and **(N)** IL cortices. Number of animals used in volumetric assessments was: 11 younger animals (RM cluster GP=7 and BP=4; WM cluster mPFC GP=8 and BP=3); and 25 older animals (reference memory cluster – mPFC: GP=13-12 and BP=12-8; working memory cluster – mPFC: GP=11-9 and BP=14-12). Continuous lines in **E**, **F**, **G**, **H**, **K**, **L**, **M** and **N** are Gaussian fits. Error bars represent SEM; $*p < 0.05$; $** \ p < 0.01$; $***p < 0.001$.

5. Discussion

The main goal of the present study was to describe HPC- and mPFC-related volumetric changes occurring with normal aging, and to characterize them in relation to cognitive status, using stereological analysis and water maze-based tasks as tools to ascertain regional volumetric alterations and cognitive performance, respectively.

Collectively, and according to our previous work (Mota *et al.*, 2018), we demonstrate the presence of age-related deficits in memory tests, and a heterogeneous performance of both young adult and older rats in working and reference memory and behavioral flexibility tasks. We were able to correlate these behavioral differences, specifically the performance in the working and reference memory tasks, with volume measurements in the HPC and mPFC. Our data revealed that younger GPs had, compared with BPs, increased HPC and mPFC volumes. In contrast, in older animals this difference was reversed, with GPs presenting lower volume than BPs on both brain structures. Interestingly, while reference memory performance in both groups correlates with volumetric alterations in the HPC and mPFC, the working memory performance was only related with alterations in the mPFC area.

It is well established that age-related memory impairments are not related with neuronal loss (Rapp & Gallagher, 1996; Rasmussen *et al.*, 1996; Merril *et al.*, 2001), rather, it has been ascribed to volume changes and altered morphology of neurons in areas implicated in cognitive abilities (de Brabander *et al.*, 1998; Markham & Juraska, 2002; Driscoll *et al.*, 2003 and 2006; Dickstein *et al.*, 2013; reviewed by Burke & Barnes, 2006; Dickstein *et al.*, 2007). Moreover, there is some evidence of an existing relationship between neuronal remodeling and volumetric alterations (Cerqueira *et al.*, 2005 and 2007). Since the HPC and mPFC are critical structures for learning and memory, these two structures have been the focus of a productive line of research to determine the mechanisms of the aging brain. In this regard,

imaging studies have identified HPC and mPFC subfields that are selectively affected by aging, whereas volumetric alterations in these structures have been coupled to the status of spatial learning (Rasmussen *et al.*, 1996; Rapp *et al.*, 1996 and 1999; Van Petten, 2004). The implicit link for aging is, of course, an existence of volumetric atrophy which impairs function. However, this link is not as trivial as it appears. These contradictory findings could be justified by the fact that most of the studies performed in humans relay on humans with pathology and that the heterogeneity observed in the aging brain has not been a focus of attention in previous volumetric studies.

Also, in contrast to the common and intuitive belief that larger HPC are better, some studies surprisingly reported a negative correlation between cognitive abilities and HPC volume (Chantôme *et al.*, 1999; Van Petten, 2004; Molnár & Kéri, 2014). Molnár & Kéri (2014) demonstrated that in individuals with Fragile X Syndrome with known abnormal pruning, there is an inverse correlation between HPC volume and cognitive performance: larger HPC was associated with worse general memory, which is not present in age-matched individuals without pruning deficits. In addition, a meta-analysis from Van Petten (2004) demonstrated also a negative correlation between HPC size and memory in young adults, whereas the correlation was positive in older participants. According to some authors, this paradoxical negative correlation might be related to incomplete synaptic pruning during childhood and adolescence, which refers to the elimination of unnecessary neurons and synapses to achieve more economic information processing (Foster *et al.*, 1999; Pohlack *et al.*, 2014); while other authors, in a more rudimentary way, explained this by the amount of effort provided by the subject: those who had difficulties performing the task tended to make greater effort and thus activated the associated structure(s) more, while those for whom the task was less difficult used more efficient strategies and needed less effort (Parks *et al.*, 1988). Hence, larger hippocampus might be less optimal for learning and memory. Our own previous findings have shown that larger dendritic trees were associated with best cognitive performances in young subjects, whereas the opposite was true for older individuals, where decreased neuronal complexity, driven by a more efficient autophagy-dependent dendritic pruning, was associated with a healthier cognitive aging process (Mota *et al.*, 2018).

First of all, it is important to mention that the present results document an enlargement of the HPC and mPFC volumes in the older rats when compared to the youngster counterparts. These data are against the majority of studies reported so far. For instance, Hamezah and collaborators in 2017, using magnetic resonance imaging, showed that the mPFC and HPC volumes were smaller in 27-month-old rats than in 14-month-old rats.

Nevertheless, in accordance with our results, Ojo B. *et al.*, (2013) have shown that the volume of DHPC CA3 increases with age. We cannot exclude, however, that alterations in astrocytic number, axonal myelination, and extracellular volume, could influence the observed differences between young and aged individuals. Besides, our data was not normalized to the whole-brain volume as widely described in human studies (reviewed by van Petten C, 2004). Nevertheless, since our main goal was to understand the inter-individual differences between younger and older animals, and between GPs and BPs within each age category, age-related volume alterations will probably not interfere with our interpretations.

Thus, bearing in mind the categorization of the animals as GPs and BPs, the performance of both older and younger animals in the reference memory task (HPC-dependent task) correlates with volumetric alterations in HPC and mPFC area, while the performance in the working memory tasks (mPFC-dependent task), correlates solely with mPFC volumetric alterations. Moreover, our work revealed that young animals classified as GPs, when compared to the bad ones, presented, in general, an increased HPC and mPFC volume. Surprisingly, old animals classified as GPs showed the opposite, as they presented a decreased volume on both brain structures, whereas the bad ones presented an increased volume. These results fit with the morphological data previously reported by Mota *et al.*, 2018 for young and old animals and translate in equivalent volumetric changes in HPC and mPFC areas. While in young animals, better cognitive performance was associated with longer dendritic trees and higher levels of synaptic markers, this association was exactly the opposite in the older group, in which better performance was associated with shorter dendritic branches and lower levels of synaptic markers (Mota *et al.*, 2018).

Moreover, our data further strengths the evidence that, in old animals with deficits in HPC and mPFC-dependent tasks, the larger dendritic trees observed result from impaired autophagy, leading to dendritic pruning deficits and consequently impaired memory (Mota *et al.*, 2018). In line with this and taking into account the study of Molnár & Kéri (2014), it is plausible to suggest that the inverse correlation, between HPC volume and cognitive performance in the elderly, might be attributed to age-associated dendritic pruning deficits leading to larger, less efficient, dendritic trees and consequently increased volume. Of note, in our work, the inverse correlations between volume/dendritic tree length and cognitive performance are not present in the group of younger adults (in which an opposite trend is observed), pointing to an age-related phenomenon.

Furthermore, volumetric alterations in the HPC only occurred in the dorsal pole of the HPC axis. These results are in line with the different functions of the two hippocampi: the DHPC is involved in memory and cognitive processing, whereas the VHPC processes information related to the emotional and homeostatic states of the animal (Fanselow & Dong, 2010; McHugh *et al.*, 2011). Given that the integrity

of the HPC circuitries is crucial for the numerous functions ascribed to this region of the brain, it is conceivable that structural alterations in the HPC might lead to circuit disruption and to the compromise of the successful performance in the spatial memory tasks (Morris, 1984). While the DG is thought to contribute to the formation of new memories, the CA3 plays an important role in the encoding of new spatial information in short-term memory whereas the CA1 is important for representing and remembering spatial information (Amaral *et al.*, 2007; Kesner *et al.*, 2007; Ji & Maren, 2008). Accordingly, our data showed that the volumetric alterations in young animals were observed specifically in the DG granular layer and in the stratum radiatum of CA1 both in the dorsal pole of the HPC axis. A similar result was observed for the aged animals, with alterations in the volume of the dorsal DG (molecular, granular and hilus) and dorsal CA1 (strata oriens, pyramidale and radiatum). Interestingly, no alterations in the CA3 area were observed between GPs and BPs, either in young or aged animals. Thus, it can be inferred that both the DG and CA1 were preferentially affected by age and could, therefore, be linked to alterations in spatial learning, while the CA3 region seems to be fairly resistant to the aging process. These particular data are, in one hand, in line with the research of Yang *et al.*, (2013) where they revealed that, unlike in CA1 synapses, the high frequency stimulation of the associative/commissural pathway leading to CA3 long-term potentiation is minimally affected by age; on the other hand, its against the majority of studies that documented a role for CA3 in the acquisition of spatial memory in the Morris water maze task (Steffenach *et al.*, 2002; Jo *et al.*, 2007). These data then suggest that age-related changes may not be uniform across the hippocampus, and given the complexity of this structure, whether changes are seen depends on the specific region under investigation.

The mPFC plays a key role in decision making, planning, processing of emotional stimuli, behavioral flexibility, in social interactions, as well as in working memory (Damasio, 2000; Manes *et al.*, 2002; Muller *et al.*, 2002). In the rat, the mPFC consists of three main subdivisions which are the: Cg, PL and IL cortices (Van Eden, 1985). These different subdivisions also appear to serve separate and distinct functions. For example, the Cg has been implicated in the control of actions, decision making and plays a key role in the expression of remote memory (Teixeira *et al.*, 2006), while the PL and IL area have been associated with autonomic/emotional control and have remarkably dense reciprocal connections with the HPC formation, thus being specifically implicated in working memory. (Ongur & Prince, 2000; Vertes, 2004; Gisquet-Verrrier & Delatour, 2006; Cerqueira *et al.*, 2008; Euston *et al.*, 2012). Our results showed that the behavioral correlates of mPFC volume were different for younger and older animals. Specifically, in younger animals, in general, the regional volume of mPFC positively correlates with the working memory performance, while in older rats volumetric alterations negatively correlates with the reference

memory performance. As expected, and similar to what we previously described (Mota *et al.*, 2018), the direction of changes was similarly reversed between young and aged individuals, with young GPs presenting larger and aged GPs smaller mPFC volumes, when compared with BPs. The areas responsible for the differences observed between the younger GPs and BPs animals were the PL an IL areas, which is in accordance with the role of these areas regarding the working memory performance (Gisquet-Verrrier & Delatour, 2006). By contrast, in older animals volumetric alterations was essentially described in the Cg area. This area has been associated with remote memory and in conformity with this, volumetric changes of Cg area correlates with reference memory performance (long-term memory task) (Teixeira *et al.*, 2006). Given this, and similarly to what happens in the hippocampus, the age-related volumetric changes may be different across the mPFC.

In summary, our findings show some important changes that occur in the rat HPC and mPFC with normal aging, and these changes are related selectively to different cognitive performances, regarding HPC or mPFC-dependent memory tasks.

Taken together, these results strongly support that volumetric alterations in HPC and mPFC are pivotal in mediating age effects in the brain and fits with our previous data regarding neuron morphology (Mota *et al.*, 2018). A summary of the volumetric data is presented in figure 4. Both for volumetric and morphological data, the theory that "bigger is always better" for the performance of young animals it's true, however, and apparently counterintuitive, for aged animals, "smaller is better".

Hopefully, our work will provide a novel hypothesis to understand the individual differences observed with aging not only in rodents but also in humans. Understanding the mechanisms behind volumetric and dendritic changes and how these changes contribute to the brain's capacity for memory, will aid in the development of new therapeutic avenues and to prevent or restore cognitive impairments towards a successful brain aging.

Young animals **Old animals**

mPFC HPC mPFC HPC

Good Performers

mPFC HPC mPFC HPC

Bad Performers
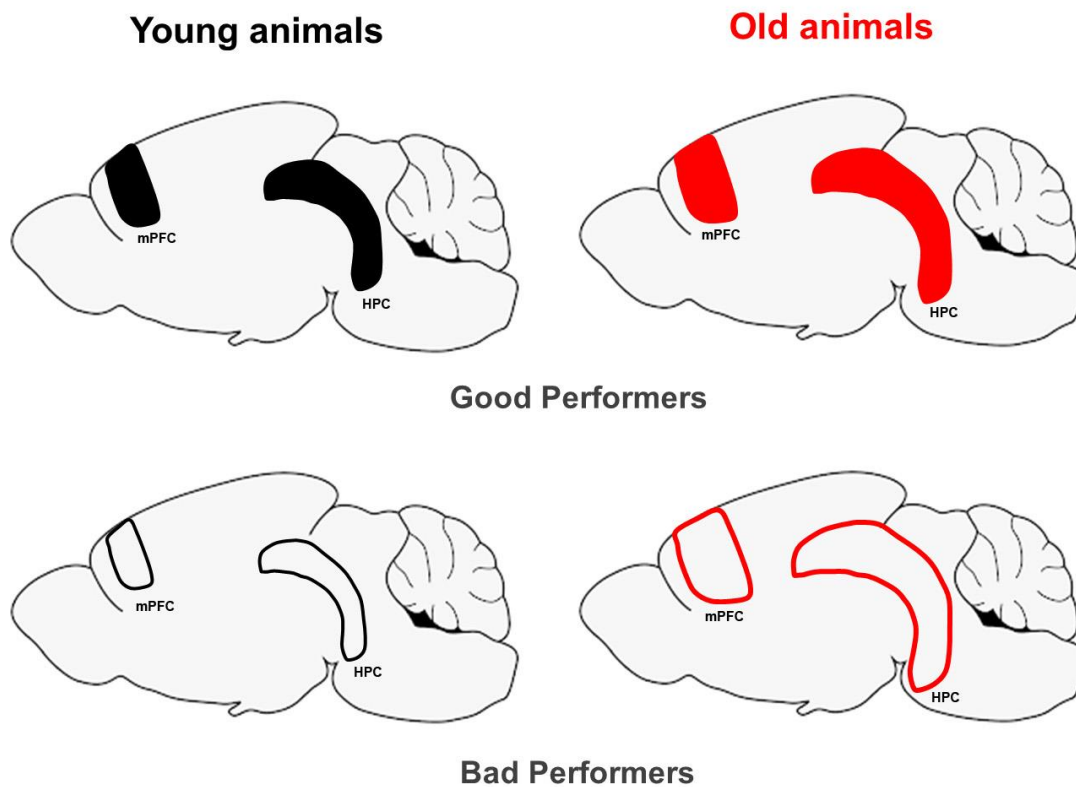
Figure 4| Schematic representation of the volumetric alterations in the HPC and mPFC in cognitive aging. In younger animals "bigger is better"; better cognitive performance was associated with increased HPC and mPFC volumes. In older animals, it seems that "smaller is better". BPs have increased HPC and mPFC volumes.

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Table 1| Results of two-way ANOVA, repeated measures and *t*-test regarding the behavioral data obtained from younger and older animals.

	Behavioral assessment (Fig. 1a,b,c)		Performance			Age			Interaction		
		df	F	P	$\eta\rho^2$	F	P	$\eta\rho^2$	F	P	$\eta\rho^2$
Two-way ANOVA	Working Memory	1,42	**21.744**	**<0.0005**	**0.341**	**30.343**	**<0.0005**	**0.419**	0.711	0.404	0.017
	Reference Memory	1,42	**64.239**	**<0.0005**	**0.605**	**46.205**	**<0.0005**	**0.524**	0.006	0.938	0.000
	Behavioral Flexibility										
	New quadrant	1,40	**63.223**	**<0.0005**	**0.612**	0.001	0.978	0.000	**12.420**	**0.001**	**0.237**
	Old quadrant	1,40	1.729	0.196	0.041	0.302	0.586	0.007	0.146	0.705	0.004
Repeated measures	**Working Memory** *(number of GP, BP)*										
		df	F	P	$\eta\rho^2$						
	Younger animals (GP n=10, BP n=4)	1,12	**5.508**	**0.037**	**0.315**						
	Older animals (GP n=15, BP n=17)	1,30	**27.613**	**<0.0005**	**0.479**						
	Reference Memory *(number of GP, BP)*										
	Younger animals (GP n=9, BP n=5)	1,12	**32.319**	**<0.0005**	**0.729**						
	Older animals (GP n=18, BP n=14)	1,30	**50.187**	**<0.0005**	**0.625**						
t-test	**Behavioral Flexibility - New Quadrant (number of GP, BP)**										
		df	t	P	d						
	Younger animals (GP n=7, BP n=5)	10	**4.100**	**0.002**	**2.453**						
	Older animals (GP n=8, BP n=24)	30	**9.201**	**<0.0005**	**0.463**						
	Behavioral Flexibility - Old Quadrant (number of GP, BP)										
	Younger animals (GP n=7, BP n=5)	10	-0.774	0.457	0.463						
	Older animals (GP n=8, BP n=24)	28	-2.024	0.052	0.663						

* p<0.05; ** p<0.01;*** p<0.001.

Table 2| Results of *t*-test analysis, between young and old animals, regarding the volumetric alterations in the HPC and mPFC.

	Young vs Old animals			
	df	*t*	*P*	*d*
Volume of HPC (Fig. 2a)				
Total HPC	**37**	**3.966**	**0.0003**	**1.347**
Dorsal	**37**	**3.734**	**0.0006**	**1.268**
Ventral	40	1.118	0.270	0.259
Volume of the subdivisions of DHPC (Fig. 2b)				
DG				
Molecular layer	**37**	**3.973**	**0.0003**	**1.350**
Granular layer	38	1.420	0.164	0.479
Hilus	**36**	**6.464**	**<0.0005**	**1.646**
CA3				
Stratum oriens	37	1.307	0.199	0.444
Stratum pyramidale	38	0.780	0.440	0.263
Stratum radiatum	**37**	**3.049**	**0.004**	**1.036**
CA1				
Stratum oriens	**41**	**3.303**	**0.002**	**1.097**
Stratum pyramidale	41	0.668	0.508	0.222
Stratum radiatum	**41**	**3.167**	**0.003**	**1.051**
Volume of mPFC (Fig. 3a)				
Total	**27**	**2.958**	**0.006**	**0.892**
Cg	29	-0.320	0.751	0.090
PL	**32**	**4.783**	**<0.0005**	**1.340**
IL	**32**	**2.113**	**0.043**	**0.775**
Volume of the subdivisions of mPFC (Fig. 3b)				
Cg				
Layer I	34	1.232	0.226	0.329
Layer II	28	-0.693	0.494	0.192
Layer III	30	-0.612	0.545	0.176
PL				
Layer I	**31**	**5.240**	**<0.0005**	**1.451**
Layer II	**31**	**3.698**	**0.001**	**1.018**
Layer III	**32**	**4.205**	**<0.0005**	**1.190**
IL				
Layer I	**29**	**2.801**	**0.009**	**0.750**
Layer II	32	1.351	0.186	0.495
Layer III	32	1.601	0.119	0.587

*** p < 0.001; ** p< 0.01; * p < 0.05.

Table 3| List of correlations between the performance in reference memory task and the volume of HPC formation.

			Young animals	Old animals
			Reference memory	
HPC formation	Total HPC	Pearson Correlation	0.710··	-0.649···
		Sig. (2-tailed)	0.007	0.0003
		N	13	26
	Dorsal HPC	Pearson Correlation	0.740··	-0.549··
		Sig. (2-tailed)	0.004	0.004
		N	13	26
	Ventral HPC	Pearson Correlation	0.180	-0.159
		Sig. (2-tailed)	0.555	0.392
		N	13	31
DHPC subdivisions	DG - Molecular layer	Pearson Correlation	0.547	-0.507··
		Sig. (2-tailed)	0.053	0.008
		N	13	26
	DG - Granular layer	Pearson Correlation	0.708··	-0.520··
		Sig. (2-tailed)	0.007	0.005
		N	13	27
	DG - Hilus	Pearson Correlation	0.258	-0.470·
		Sig. (2-tailed)	0.395	0.013
		N	13	27
	CA3 stratum oriens	Pearson Correlation	0.484	-0.061
		Sig. (2-tailed)	0.094	0.766
		N	13	26
	CA3 pyramidal layer	Pearson Correlation	0.636·	0.004
		Sig. (2-tailed)	0.019	0.983
		N	13	27
	CA3 stratum radiatum	Pearson Correlation	0.544	-0.237
		Sig. (2-tailed)	0.055	0.243
		N	13	26
	CA1 stratum oriens	Pearson Correlation	0.554	-0.417·
		Sig. (2-tailed)	0.050	0.022
		N	13	30
	CA1 pyramidal layer	Pearson Correlation	-0.165	-0.365·
		Sig. (2-tailed)	0.589	0.047
		N	13	30
	CA1 stratum radiatum	Pearson Correlation	0.639·	-0.495··
		Sig. (2-tailed)	0.019	0.005
		N	13	30

*** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.

110

Table 4| Results of two-way ANOVA on the data obtained from young and old animals.

		Performance			Age			Interaction		
	df	F	P	ηρ²	F	P	ηρ²	F	P	ηρ²
Volumetric analysis										
Hippocampus (Fig. 2c,d)										
Total	1,38	1.302	0.262	0.036	**26.200**	**<0.0005**	**0.428**	**13.870**	**0.001**	**0.284**
Dorsal	1,38	0.953	0.336	0.027	**22.590**	**<0.0005**	**0.392**	**13.084**	**0.001**	**0.272**
Ventral	1,43	0.049	0.825	0.001	0.627	0.433	0.015	0.034	0.854	0.001
Dorsal – DG molecular layer	1,38	0.502	0.483	0.014	**22.303**	**<0.0005**	**0.389**	**9.195**	**0.005**	**0.208**
Dorsal – DG granular layer	1,39	0.859	0.360	0.023	3.954	0.054	0.099	**11.218**	**0.002**	**0.238**
Dorsal – DG hilus	1,39	2.209	0.146	0.058	**29.007**	**<0.0005**	**0.455**	3.581	0.067	0.090
Dorsal – CA3 stratum oriens	1,38	0.000	0.996	0.000	1.876	0.179	0.051	1.094	0.303	0.030
Dorsal – CA3 pyramidal layer	1,39	0.179	0.675	0.005	0.720	0.402	0.020	0.908	0.347	0.025
Dorsal – CA3 stratum radiatum	1,38	0.465	0.500	0.013	**10.961**	**0.002**	**0.238**	3.349	0.076	0.087
Dorsal – CA1 stratum oriens	1,42	0.025	0.874	0.001	**17.466**	**<0.0005**	**0.309**	**12.858**	**0.001**	**0.248**
Dorsal – CA1 pyramidal layer	1,42	3.354	0.075	0.079	0.836	0.366	0.021	1.952	0.170	0.048
Dorsal – CA1 stratum radiatum	1,42	0.991	0.326	0.025	**18.849**	**<0.0005**	**0.326**	**15.909**	**<0.0005**	**0.290**
mPFC – Cluster based on RM (Fig. 3c,d)										
Total	1,27	0.280	0.601	0.010	**7.778**	**0.010**	**0.224**	3.086	0.090	0.103
Cg area	1,29	2.274	0.142	0.073	0.018	0.893	0.001	**4.227**	**0.049**	**0.127**
PL area	1,30	0.153	0.689	0.005	**14.644**	**0.001**	**0.328**	2.247	0.144	0.070
IL area	1,30	1.932	0.175	0.060	**5.513**	**0.026**	**0.155**	0.684	0.415	0.022
mPFC – Cluster based on WM (Fig. 3i,j)										
Total mPFC	1,27	0.019	0.891	0.001	**7.276**	**0.012**	**0.212**	**4.877**	**0.036**	**0.153**
Cg area	1,29	0.008	0.929	0.000	0.002	0.967	0.000	1.276	0.268	0.042
PL area	1,30	0.014	0.906	0.000	**15.196**	**0.001**	**0.336**	**4.766**	**0.037**	**0.137**
IL area	1,30	1.510	0.229	0.048	**6.185**	**0.019**	**0.171**	1.309	0.262	0.042

Two-way Anova

*** p < 0.001; ** p< 0.01; * p < 0.05.

Table 5| Results of *t*-test analysis on the data obtained from young GPs and BPs and old GPs and BPs, regarding the volumetric alterations in the HPC and mPFC.

	Young animals (GPs vs BPs)				Old animals (GPs vs BPs)			
	df	*t*	*P*	*d*	*df*	*t*	*P*	*d*
Volumetric analysis								
Hippocampus (Fig. 2,c,d)								
Total	**11**	**-2.343**	**0.039**	**1.453**	**24**	**3.915**	**0.001**	**1.564**
Dorsal	**8**	**-3.830**	**0.005**	**1.946**	**24**	**3.570**	**0.002**	**1.430**
Ventral	11	0.260	0.800	0.153	29	0.323	0.749	0.123
Dorsal – DG molecular layer	**11**	**-2.408**	**0.035**	**1.486**	**24**	**2.887**	**0.008**	**1.150**
Dorsal – DG granular layer	**7**	**-4.001**	**0.005**	**2.011**	**25**	**3.346**	**0.003**	**1.332**
Dorsal – DG hilus	11	-0.913	0.381	0.524	**25**	**2.553**	**0.017**	**1.015**
Dorsal – CA3 stratum oriens	11	-1.774	0.104	1.077	24	0.834	0.413	0.323
Dorsal – CA3 pyramidal layer	**11**	**-2.413**	**0.034**	**1.409**	25	0.419	0.679	0.160
Dorsal – CA3 stratum radiatum	11	-1.284	0.225	0.776	24	1.989	0.058	0.781
Dorsal – CA1 stratum oriens	11	-1.945	0.078	1.242	**28**	**3.294**	**0.003**	**1.182**
Dorsal – CA1 pyramidal layer	11	0.855	0.754	0.179	**28**	**2.897**	**0.007**	**1.080**
Dorsal – CA1 stratum radiatum	**11**	**-2.291**	**0.043**	**1.432**	**28**	**4.192**	**<0.0005**	**1.539**
mPFC – Cluster based on RM (Fig. 3c,d)								
Total mPFC	9	-1.659	0.131	1.009	15	1.920	0.074	0.809
Cg area (total)	9	-0.716	0.492	0.479	**20**	**2.693**	**0.014**	**1.185**
Cg layer I	**9**	**-3.062**	**0.014**	**1.791**	**23**	**2.157**	**0.042**	**0.866**
Cg layer II	9	-0.464	0.654	0.314	**20**	**2.764**	**0.012**	**1.189**
Cg layer III-VI	9	-0.208	0.840	0.137	**20**	**2.690**	**0.014**	**1.182**
PL area (total)	9	-1.489	0.171	0.851	21	1.471	0.156	0.618
PL layer I	9	-0.584	0.573	0.368	15	0.849	0.409	0.349
PL layer II	9	-0.687	0.510	0.448	19	0.875	0.393	0.362
PL layer III-VI	9	-1.638	0.136	0.932	21	1.575	0.130	0.659
IL area (total)	**9**	**-2.410**	**0.039**	**1.582**	13	-0.446	0.660	0.190
IL layer I	9	1064	0.315	0.709	14	-0.427	0.675	0.175
IL layer II	9	0.018	.0987	0.011	14	0.081	0.936	0.033
IL layer III-VI	**9**	**-2.722**	**0.024**	**1.894**	14	-0.484	0.636	1.223
mPFC – Cluster based on WM (Fig. 3i,j)								
Total mPFC	**9**	**3.635**	**0.005**	**2.897**	18	-1.767	0.094	0.800
Cg area (total)	9	1.653	0.133	1.230	20	-0.969	0.344	0.409
Cg layer I	9	2.103	0.065	1.359	23	-1.521	0.142	0.608
Cg layer II	9	1.706	0.122	1.307	20	-0.804	0.431	0.339
Cg layer III-VI	9	1.141	0.284	0.767	20	-0.880	0.389	0.371
PL area (total)	**9**	**3.719**	**0.005**	**2.930**	21	-1.855	0.078	0.777
PL layer I	**9**	**3.007**	**0.015**	**1.982**	**21**	**-2.149**	**0.043**	0.881
PL layer II	**9**	**2.823**	**0.020**	**2.373**	21	-1.277	0.215	0.530
PL layer III-VI	**9**	**2.975**	**0.016**	**2.240**	21	-1.605	0.123	0.673
IL area (total)	**9**	**2.620**	**0.028**	**1.920**	21	0.070	0.945	0.029
IL layer I	9	0.914	0.385	0.669	12	1.028	0.325	0.451
IL layer II	9	1.891	0.091	1.150	21	-0.160	0.874	0.066
IL layer III-VI	9	1.762	0.112	1.318	21	-0.585	0.565	0.247

t-test

*** p < 0.001; ** p< 0.01; * p < 0.05.

Table 6| List of correlations between the performance in spatial working and reference memory task and the volume of mPFC.

			Young animals		Old animals	
			WM	**RM**	**WM**	**RM**
mPFC	**Total**	Pearson Correlation	**0.755**··	0.527	-0.405	**-0.520**·
		Sig. (2-tailed)	**0.007**	0.096	0.077	**0.019**
		N	**11**	11	20	**20**
	Cg	Pearson Correlation	**0.814**··	0.413	-0.236	**-0.571**··
		Sig. (2-tailed)	**0.002**	0.207	0.290	**0.006**
		N	**11**	11	22	**22**
	PL	Pearson Correlation	**0.668**·	0.408	-0.344	-0.358
		Sig. (2-tailed)	**0.025**	0.213	0.108	0.094
		N	**11**	11	23	23
	IL	Pearson Correlation	0.387	**0.643**·	-0.106	-0.016
		Sig. (2-tailed)	0.240	**0.033**	0.631	0.943
		N	11	**11**	23	23
Cg	**Layer I**	Pearson Correlation	0.384	**0.650**·	-0.287	**-0.557**··
		Sig. (2-tailed)	0.244	**0.030**	0.164	**0.004**
		N	11	**11**	25	**25**
	Layer II	Pearson Correlation	0.596	0.145	-0.171	**-0.542**··
		Sig. (2-tailed)	0.053	0.671	0.447	**0.009**
		N	11	11	22	**22**
	Layer III-VI	Pearson Correlation	**0.781**··	0.300	-0.225	**-0.535**·
		Sig. (2-tailed)	**0.005**	0.370	0.313	**0.010**
		N	**11**	11	22	**22**
PL	**Layer I**	Pearson Correlation	0.433	0.134	**-0.453**·	-0.334
		Sig. (2-tailed)	0.183	0.696	**0.030**	0.119
		N	11	11	**23**	23
	Layer II	Pearson Correlation	0.361	0.126	-0.215	-0.303
		Sig. (2-tailed)	0.275	0.712	0.324	0.160
		N	11	11	23	23
	Layer III-VI	Pearson Correlation	**0.678**·	0.461	-0.288	-0.329
		Sig. (2-tailed)	**0.022**	0.154	0.183	0.125
		N	**11**	11	23	23
IL	**Layer I**	Pearson Correlation	-0.145	-0.351	0.064	0.010
		Sig. (2-tailed)	0.670	0.290	0.773	0.963
		N	11	11	23	23
	Layer II	Pearson Correlation	0.057	-0.081	-0.052	0.017
		Sig. (2-tailed)	0.868	0.812	0.814	0.940
		N	11	11	23	23
	Layer III-VI	Pearson Correlation	0.394	**0.702**·	-0.201	-0.037
		Sig. (2-tailed)	0.230	**0.016**	0.357	0.868
		N	11	**11**	23	23

*** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.

Adulthood cognitive training selectively prevents age-related memory impairments in the rat

Mota C, Pinto V, Sousa N, Sousa JC & Cerqueira JJ

Adulthood cognitive training selectively prevents aged-related memory impairments in the rat

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1. Abstract

In general, old rats present memory impairments. Since cognitive training can enhance memory function, here we tested if it is also able to prevent age-associated deficits. The Hole board (HB) test was used as a cognitive-training tool for hippocampal (HPC)-dependent spatial reference memory, and animals were trained in four sets of training paradigms, designed to assess the effects of cognitive training, but also to explore the influence of variables such as the optimal time to start the training task. Exposure to only one training session at 10 months of age, but not at 15 or 20 months, was sufficient to improve reference memory performance after one year. By contrast, this training paradigm was unable to induce any change in working memory or behavioral flexibility skills of the same animals, suggesting that its effects are task specific. When we analyzed the structural HPC alterations underpinning these behavioral improvements, differences were only detected when the animals were divided into good and bad performers (GPs, BPs). Under these analysis conditions, the group of BPs untrained old animals had the biggest HPC and the longest DG and CA1 apical dendritic trees, in line with our previous observations that poor cognitive performance in the old age was associated with pruning deficits and longer dendritic arborizations.

In conclusion, these results suggest that cognitive training is long lasting in time, mainly triggering synaptic plasticity within the brain circuits involved in the specific training paradigm. Importantly, this study suggests that even a relatively short period of cognitive enrichment is sufficient to improve the ability to recover from age-induced impairments, which might prove relevant for successful aging. These promising findings underline the existence of continuous functional plasticity, which brings optimism about the possibility of improving cognitive function in old age.

2. Introduction

The aging process presents a great inter-individual variability. Nevertheless, it is generally accepted that aging is mostly associated with decreasing cognition (Erickson & Barnes, 2003; Singh-Manoux *et al.*, 2012; Santos *et al.*, 2013). Since memory impairments impose a substantial burden for those affected, the development of interventions to maintain and optimize cognitive functioning has skyrocketed.

Several studies have tried to tackle on strategies to prevent cognitive decline in old age, suggesting interventions based on nutritional improvements (Richards *et al.*, 2002), physical exercise (Tanigawa *et al.*, 2014) and cognitive training (Nouchi *et al.*, 2012; Jiang *et al.*, 2016).

In humans, cognitive training, both early and later in life, through engagement in intellectually stimulating activities, is associated with better cognitive functioning, as it postpones or attenuates cognitive decline,

and ameliorates cognitive deficits (Wilson *et al.*, 2002; reviewed by Frick & Benoit, 2010 and Milgram *et al.*, 2006; Belleville & Bherer, 2012; Rebok *et al.*, 2014). These effects could be explained by the cognitive reserve hypothesis, which rests on the ability of the brain to compensate for pathological changes associated with aging, depending on the previous stage of intellectual capability (Whalley *et al.*, 2004). As such, cognitive training induces increased dendritic length of neurons (Jacobs *et al.*, 1993), increased gray matter volumes in the hippocampus (HPC) (Maguire *et al.*, 2006; Cannonieri *et al.*, 2007), increased cortical and subcortical volumes (Seider *et al.*, 2016) and resulted in important changes of brain activity (Hempel *et al.*, 2004; Olesen *et al.*, 2004).

On the other hand, in rodents, the beneficial effects of cognitive training have also been associated with physical stimulation, which together is often referred to as environmental enrichment (EE) (Fischer, 2016).

Environmentally enriched rodents are typically socially housed in large groups and exposed to a variety of stimulating objects that can provide both cognitive stimulation (e.g., toys, tunnels, dwellings) and physical exercise (e.g., running wheels). For example, Vicens *et al.*, (1999, 2002) found that a water maze training task, performed in 6-month-old mice, led to an improved performance in the water maze later at 10 and 18 months of age. More recently, Galeano *et al.*, 2015 showed that life-long EE was able to rescue memory deficits in control aged rats and in aged rats that were subjected to asphyxia at birth. Additionally, several research groups have systematically shown that EE induces synaptogenesis (Greenough & Volkmar, 1973; Rampon *et al.*, 2000), neurogenesis (Kempermann *et al.*, 2002), cortical thickening (Mohammed *et al.*, 2002) and dendritic branching (Faherty *et al.*, 2003; Kolb *et al.*, 2003; Bindu *et al.*, 2007). Interestingly, these anatomical changes correlate with functional alterations such as increased HPC long-term potentiation (LTP) (Artola *et al.*, 2006, Stein *et al.*, 2016).

Since the HPC is particularly vulnerable to aging (Mora *et al.*, 2007; Mota *et al.*, 2018), and the Hole Board (HB) task is a highly demanding task which works as a stimulus for spatial reference memory (HPC-dependent task) (Van der Staay, 1999; Depoortère *et al.*, 2010), we hypothesized that the HB task would have the ability to enhance memory function in old animals. To this end, cognitive training was performed in rats of different ages that were later assessed for cognitive performance in a battery of water maze tests. In addition, we tested if there was a relationship between HPC-dependent HB training and changes in HPC morphology and neuron number.

3. Methods
3.1. Animals

All procedures were carried out in accordance with local regulations (Decreto-Lei n.º 113/2013) and European Union Directive 2010/63/EU on animal care and experimentation. Animal facilities and the people directly involved in animal experiments were certified by the Portuguese regulatory entity – DGAV (Direção-Geral de Alimentação e Veterinária). All protocols were approved by the Ethics Committee of the Life and Health Sciences Research Institute (ICVS). All the male Wistar Han rats (Charles River Laboratories, Barcelona, Spain) used in the study were housed in groups of 2 and maintained under standard laboratory conditions: artificial 12h light/dark cycle (lights on from 8:00 a.m. to 8:00 p.m.); room temperature 22°C; *ad libitum* access to food and water. All behavioral testing was conducted during the daytime. A total of 82 male rats were used in this study divided in 3 sets of experiments, with the only difference between them being the age at which cognitive training was administered (10 months, 15 months, 20 months). Animals of each experiment were randomly allocated to the group that performed cognitive training (HB group: 10 months n=22, 15 months n=9, 20 months n=11) or the control group that was put in the hole board apparatus and rewarded independently of any spatial learning (pseudo-Hole Board group: 10 months n=19, 15 months n=12, 20 months n=9). After training, all animals waited to reach 22 months of age when they were behaviorally characterized in a battery of water-maze based tests to assess cognition. Two months later (24 months), animals were sacrificed and their brains removed for further analysis. Brains of a subset of control and cognitively trained old animals, from the 10 months experimented exclusively, were used to perform morphological analysis (3D neuron reconstruction) and stereological analysis (volumes estimations and neuronal number). As there were no differences in the behavior of animals trained at other ages, their brains were not processed for the purpose of the present study and results will not, therefore, be presented. Details on the experimental design are depicted in figure 1.
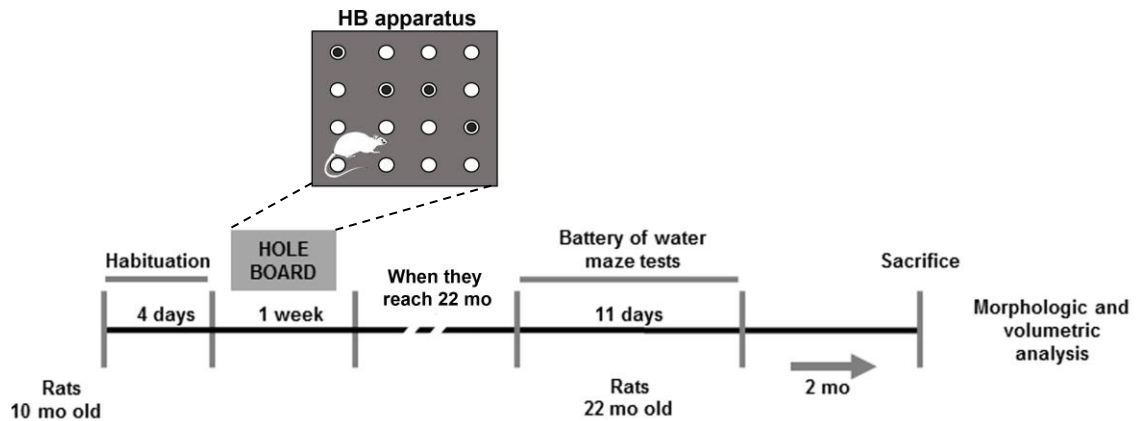
118

Figure 1| Experimental design and timeline for behavioral tests performed by Pseudo-HB and HB groups. (mo: months).

3.2.   Behavioral assessment

Cognitive training - HB Task

The HB food-retrieval task is a highly demanding task and works as a stimulus for spatial reference memory (HPC-dependent task). This task allows the assessment of working (WM) and reference memory (RM) performance. The HB apparatus consisted of an open arena (square board), 70x70cm, with opaque walls and 16 holes of 3.5cm diameter (Fig. 1). Four of these holes were baited with rewards (Cheerio®-like cereals) with constant trial-to-trial disposition for each animal, but random distribution patterns between different animals. To prevent the animals from using scent to find rewards, a few cereals were ground and scattered over the rest of the holes. Two days before the beginning of the task and during the 5 days of the task, the animals entered a regimen of food deprivation having access to food only 1h per day. One day before the task, a reward cereal was given to the animals so that they could get used to its smell and taste. Daily sessions of 1 trial per day were given to each animal for 5 consecutive days. At the beginning of each trial, the animal was placed on the center of the platform and allowed to explore. The trial ended whenever the animal found the 4 rewards, or after a maximum of 30min. The number of right answers (nose poke on the holes that contained rewards), wrong answers (WA) (nose poke on empty holes), eaten rewards, repetitions after reward (RAR) (nose poke on correct holes that no longer had the reward), time to find the first reward and the total time of the trial were registered, for each animal and each trial. WM errors, a performance directly related to WM, corresponded to the number of RAR and RM errors, a measure related to RM status, were given by the number of WA. The pseudo-HB group was subjected to the same experimental conditions except that the pellets were exposed in the open arena.

119

Water maze tests

The cognitive status of all animals was assessed based on their performance in a series of tasks using the water maze. Animals were tested during 8 days in 3 tests designed to assess different cognitive domains: WM, RM and behavioral flexibility (BF) (Cerqueira *et al.*, 2007). The apparatus consisted of a large circular black pool (170 cm diameter), filled to a depth of 31 cm with water (at 22°C), which was divided in 4 equal-sized quadrants by imaginary lines. During the execution of the test, a submerged cylindrical black platform (12 cm diameter, 30 cm high) was hidden 2 cm below the water surface at the center of one of the quadrants. The room was dimly lit and extrinsic visual clues were glued to the walls surrounding the tank and kept unaltered during the duration of the experiment. Data was collected using a video camera placed above the center of the pool connected to a video-tracking system (Viewpoint, Champagne au Mont d'Or, France).

Working memory task: this task is a variation of the spatial RM test (Morris, 1984) and depends on medial pre-frontal cortex (mPFC) function (Kesner, 2000). Its goal is to assess the ability of rats to learn the position of a hidden platform and to keep this information online during four consecutive trials. This test consisted of 4 days of acquisition in which the position of the platform was kept constant during the four daily trials [with a maximum of 120 seconds (s) per trial] but was altered to a different quadrant every day (such that all four quadrants are used). Thus, the animal cannot know where the platform was hidden on trial 1 of each day. Test sessions begun with rats facing the wall of the maze, being placed at one of the four different starting points (north, east, south, or west) which were different for each of the four daily trials. A trial was considered complete once the escape platform had been reached by the rat. Animals were then allowed to spend 30 s in the platform, after which they were towel-dried and allowed to rest in a holding cage some seconds before being returned to the maze. When the escape platform was not reached within 120 s, the experimenter guided the animal to the platform and an escape latency of 120 s was recorded. The length of the path described (distance swam) and the time spent to reach the platform (escaped latency) were recorded in the consecutive trials.

Reference memory task: this test is a HPC-dependent task (Morris, 1984) that evaluates the ability of the animal to learn the location of a hidden platform during four consecutive days – spatial reference memory. After working memory assessment (on days 5-7), the platform remained in the same quadrant as on the previous day (day 4) and animals were tested for an additional 3 days of tests. The remaining procedures were similar to the already described.

Behavioral flexibility task: this is a mPFC-dependent assessment and was performed after the completion of the RM task (day 8). For this test, the escape platform was moved and located in the

opposite quadrant of its previous 4 days location. All the procedures were similar to those described above. For this task, the time spent swimming in each quadrant was recorded and analyzed.

3.3.   Histological procedures

Two months after all behavioral evaluations (Mota et *al.*, 2018), 28 aged rats trained at 10 months (Pseudo HB n=15, HB n=13) were randomly selected, deeply anesthetized with sodium pentobarbital and perfused with saline. The left brain hemisphere was used for Golgi-Cox staining, while the right hemisphere was fixed in 4% paraformaldehyde solution for glycolmethacrylate inclusion. The remaining animals were sacrificed for other analyses not included in the present study. In order to ensure an unbiased analysis, slides resulting from all histological procedures were re-coded by the lab technician (not otherwise involved in the research) as soon as they were prepared. To minimize bias, codes were only broken after all data was collected and entered into the database.

Golgi-cox staining

After perfusion, brains were removed, immersed in 25 mL Golgi-Cox solution (Gibb & Kolb, 1998) (a 1:1 solution of 5% potassium dichromate and 5% mercury chloride diluted 4:10 with 5% potassium chromate) and kept in the dark at room temperature for 14 days. Brains were then transferred to a 30% sucrose solution. At this moment, brains were stored in the dark at 4°C from a minimum of 3 days to a maximum of 2 months, before being cut on a vibratome. Coronal sections (200 μm thick) were collected in 6% sucrose and blotted dry onto cleaned, gelatin-coated microscope slides. Subsequently, sections were alkalinized in 18.7% ammonia, developed in Dektol (Kodak), fixed in Kodak Rapid Fix, dehydrated through a graded series of ethanol of increasing concentrations and cleared in xylene before being covered in mounting media (Entellan New) and coverslipped. The slides were stored in the dark and exposed to the air, at room temperature, until being analyzed.

Dendritic arborizations were analyzed in the dentate gyrus (DG), cornus ammonis 3 and 1 (CA3 and CA1) of the dorsal hippocampus (DHPC). Dorsal hippocampal formation was identified according to the division described by Pinto *et al.*, 2015. The granular neurons of the DG were readily identified based on their round cell bodies, which were located in the stratum granulosum of the suprapyramidal and infrapyramidal blades. Pyramidal neurons from the DHPC (CA3 and CA1) were readily identified by their characteristic triangular soma shape, apical dendritic extension toward the pial surface and numerous dendritic spines. All neurons were chosen for reconstruction based on the criteria described by Uylings *et al.*, (1986): (i) full impregnation of the neurons along the entire length of the dendritic tree; (ii) apical

dendrite without truncated branches, except on the most superficial layer; (iii) presence of at least 3 primary basal dendritic shafts, each of which branched at least once (when applicable); (iv) relative isolation from neighboring impregnated cells that could interfere with analysis (clear somatic boundaries) (v) no morphological changes attributable to incomplete dendritic impregnation of Golgi-Cox staining. To minimize selection bias, slices containing the region of interest were randomly searched and the first 5-10 neurons fulfilling the above criteria (maximum of 3 neurons per slice) were chosen. For each selected neuron, all branches of the dendritic tree were reconstructed at 600× magnification, using a motorized microscope (Olympus BX51 Microscope with oil-objectives), attached to a camera (QImaging® Retiga-2000R digital camera, Surrey, Canada) and equipped with Neurolucida software (Microbrightfield, VT, USA). A 3-D analysis of the reconstructed neurons was performed using NeuroExplorer software (Microbrightfield, VT, USA). Dendritic morphology was examined by assessing the total dendritic length. In addition, to assess differences in the arrangement of dendritic material, a 3-D version of a Sholl analysis (Uylings & Van Pelt, 2002) was performed. For this, the number of intersections of dendrites with concentric spheres positioned at radial intervals of 20µm from the soma was recorded.

Glycolmethacrylate inclusion

Brains were removed and placed in fixative. After 4 weeks, brains were processed for stereology, according to the procedure described previously by Keuker *et al.*, (2001). Briefly, they were included in glycolmethacrylate (Tecnovit 7100; Heraeus Kulzer, Werheim, Germany) and every other microtome-cut section (30 µm) was then collected on a gelatinized slide, stained with Giemsa, and mounted with Entellan New (Merck, Darmstadt, Germany).

The DHPC was analyzed according to its main anatomical divisions: DG (including hilus, granule cell layer and molecular layer), CA3 and CA1 (strata oriens, pyramidale and radiatum) (for more details see Cerqueira *et al.*, 2005; Pinto *et al.*, 2015). The above-mentioned regions were outlined according to the atlas of Paxinos & Watson (1998), based on noticeable cytoarchitectural differences (Palomero-Gallagher & Zilles, 2004; Vogt *et al.*, 2004).

Volume estimations were performed using StereoInvestigator software (MicroBrightField, Williston, VT) and a camera (DXC390; Sony, Tokyo, Japan) attached to a motorized microscope (Axioplan 2; Zeiss, Oberkochen, Germany). Cavalieri's principle (Gundersen *et al.*, 1988) was used to assess the volume of each region, using a 4x lens. Briefly, after starting at a random position, every 20th section was used and its cross-sectional area was estimated by point counting (final magnification x112). For this, we randomly superimposed onto each area a test-point grid in which the interpoint distance, at tissue level, was 150 µm for all the three layers of the DG; 250 µm for the three layers of CA3 and CA1. The volume of the

region of interest was determined from the number of points that fell within its boundaries and the distance between the systematically sampled sections.

Average cell numbers were estimated at 600× magnification using the optical fractionator method, as described previously (West *et al.*, 1991). Briefly, beginning at a random starting position, a grid of virtual, equally-spaced 3D-boxes (30×30×15μm for CA3 and CA1, and 20×20×15μm for DG) (same grid spacing as for volume estimations) was superimposed on every 20th section; and neurons were counted whenever their nucleus came into focus within the counting box. Neurons were differentiated from other cells on the basis of nuclear size (larger in neurons than in glia cells), a prominent nucleolus, and the shape of their perikarya attributable to dendritic emergence (Peinado *et al.*, 1997).

3.4. Statistical analysis

All statistical analyses were conducted in the SPSS software package version 24 (IBM corporation, Armonk, New York). After confirmation of normality and homogeneity, appropriate statistical tests were applied to the data. To facilitate direct comparisons between different water maze tests, results of all water maze tasks were converted to a 0-100 % scale, where 0 % indicates worst possible performance (120s to reach the platform for WM and RM tests, or no time in target quadrant for the behavioral flexibility task) and 100% indicates best possible performance (0 s to reach the platform or total time in the target quadrant). HB performance was quantified according to WM or RM errors.

Clustering of animals in GPs and BPs was done using the k-means cluster analysis according to each animal performance in the last day of the water maze RM task. This performance is considered the best performance achieved during the completion of the task. Comparisons between Pseudo-HB and HB animals were done using two-tailed t-test. Repeated measures ANOVA was used to evaluate the impact of training in the HB task and in the water maze-based tests. In addition, to test the association of cognitive training and water-maze performance in hippocampal morphology, two-way ANOVAs were performed. Measures of effect size (Cohen's *d* or Eta-squared) are presented whenever appropriate. Differences were considered significant if $*p<0.5$; $**p<0.01$; $***p<0.001$.

4. Results
4.1. Cognitive training

All animal groups successfully learned the HB task, as observed by the sustained decrease in the total time to perform the test (10 mo: $F_{(2,53)}=9.263$, $p<0.0005$, $\eta\rho^2=0.306$; 15 mo: $F_{(2,16)}=3.953$, $p=0.049$,

$\eta\rho^2$=0.283; 20 mo: $F_{(2,16)}$=4.383, p=0.037, $\eta\rho^2$=0.305) (Supplementary Fig. 1a,c,e). Animals trained at 10 months of age also showed a sustained decrease in the time to find the first reward ($F_{(2,37)}$=5.597, p=0.008, $\eta\rho^2$=0.210) (Supplementary Fig. 1a), accompanied by a decrease in the number of wrong answers (reference memory indicator: $F_{(3,56)}$=6.202, p=0.001, $\eta\rho^2$=0.228) (Supplementary Fig. 1b), without a significant variation in the number of entries into holes where a reward had been previously eaten (working memory indicator: $F_{(4,84)}$=1.677, p=0.163, $\eta\rho^2$=0.074) (Supplementary Fig. 1b). When compared to this group, the sets of animals that trained at 15 and 20 months of age showed a progressive deterioration of the learning pattern. Thus, animals trained at 15 months age still presented a sustained decrease in RM errors ($F_{(3,25)}$=6.372, p=0.004, $\eta\rho^2$=0.389) (Supplementary Fig. 1d) but failed to show a reduction in the time to find the first reward ($F_{(2,19)}$=2.758, p=0.091, $\eta\rho^2$=0.216) (Supplementary Fig. 1c), while animals trained later in life (20 months) failed to show a sustained decrease both in the time to find the first reward and in the number of RM errors (Time to find de first reward: $F_{(2,18)}$=0.591, p=0.549, $\eta\rho^2$=0.056; RM errors: $F_{(3,28)}$=0.571, p=0.630, $\eta\rho^2$=0.054) (Supplementary Fig. 1e,f).

4.2. Behavioral assessment

4.2.1. Cognitive training in adulthood improves reference memory performance in old age

Analysis of the latency curves of water maze-based tests regarding WM and RM tasks (Fig. 1a,b) revealed only a significant effect of cognitive training at 10 months in RM performance (RM: $F_{(1,39)}$=6.539, p=0.015, $\eta\rho^2$=0.144), but not in WM performance ($F_{(1,39)}$=1.269, p=0.267, $\eta\rho^2$=0.032) nor behavioral flexibility (New quadrant: t(35)=0.165, p=0.870, d=0.051; Old quadrant: t(39)=0.396, p=0.694, d=0.124). Thus, HB animals trained at 10 months age, showed a higher RM learning ability in the second (Pseudo-HB: 36.01±28.28% vs HB: 53.56±23.64%; t(39)=2.165, p=0.037, d=0.678), third (Pseudo-HB: 42.80±30.65% vs HB: 63.07±21.15%; t(31)=2.493, p=0.021, d=0.781) and fourth (Pseudo-HB: 47.74±29.66% vs HB: 65.53±14.81%; t(26)=2.371, p=0.026, d=0.777) days of test (Fig. 1b). Interestingly, no effect of cognitive training was found in the sets of animals trained at older ages (15 mo, WM: $F_{(1,19)}$=0.006, p=0.940, $\eta\rho^2$=0.000 ; RM: $F_{(1,19)}$=0.001, p=0.976, $\eta\rho^2$=0.000; BF new quadrant: t(19)=1.379, p=0.184, d=0.609, BF old quadrant: t(19)=-1.169, p=0.257, d=0.524; 20 mo, WM: $F_{(1,18)}$=1.552, p=0.229, $\eta\rho^2$=0.079; RM: $F_{(1,18)}$=2.184, p=0.157, $\eta\rho^2$=0.108; BF new quadrant: t(18)=0.0507, p=0.619, d=0.227, BF old quadrant: t(18)=-0.775, p=0.449, d=0.348) (Supplementary Fig. 2).
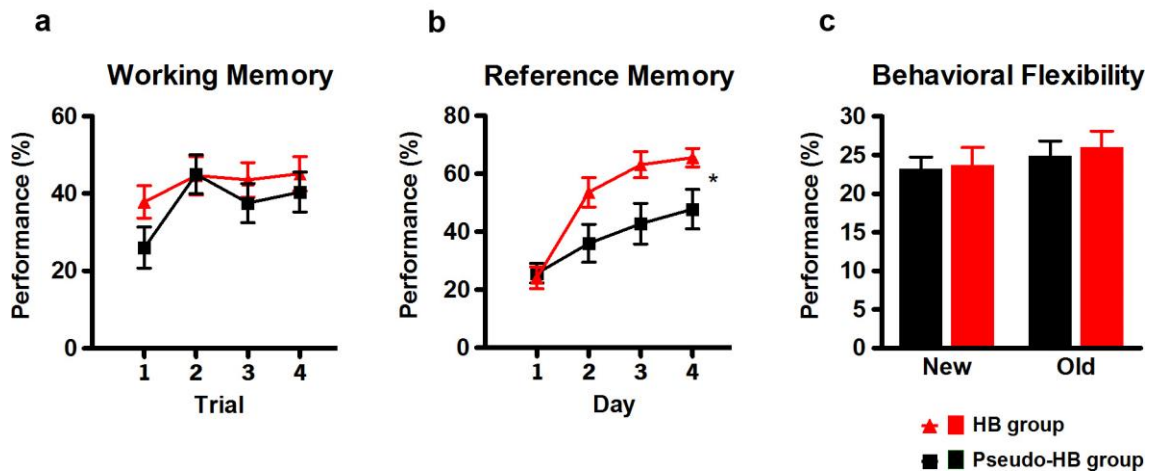
Water maze-based tests

Figure 1| Cognitive training prevents reference memory impairments in old age. A and B) Learning curves in the working **(C)** and reference memory task **(D)** of P-HB (n=19) and HB (n= 22) groups. The highest performance of the HB group in the reference memory task is easily appreciated. **C)** Results from the behavioral flexibility task. Average time spent on the four trials in the new and old imaginary quadrant is given as a percentage of the total escape latency. Error bars represent ± SEM; *p<0.05.

4.3. Structural analysis

4.3.1. Cognitive training improves hippocampal neuronal morphologies characteristic of bad-performing old animals

We next analyzed the effects of cognitive training at 10 months on the neuronal morphology of the brain region underpinning spatial RM performance (Fig. 2). Therefore, the following structural analyses were done in the subdivisions of the DHPC. In contrast to the previously shown improvements on RM performance, no differences in dendritic tree length of HPC neurons (DG granular neurons, CA3 and CA1 pyramidal neurons) were found between Pseudo-HB and HB groups (Granular neurons: $t(21)=-0.909$, $p=0.374$, $d=0.337$; CA3 apical dendrite: $t(26)=-0.935$, $p=0.359$, $d=0.350$; CA3 basal dendrite: $t(26)=0.585$, $p=0.564$, $d=0.221$; CA1 apical dendrite: $t(26)=-1.118$, $p=0.274$, $d=0.423$; CA1 basal dendrite: $t(26)=-0.975$, $p=0.339$, $d=0.368$) (Fig. 2a,b,c).
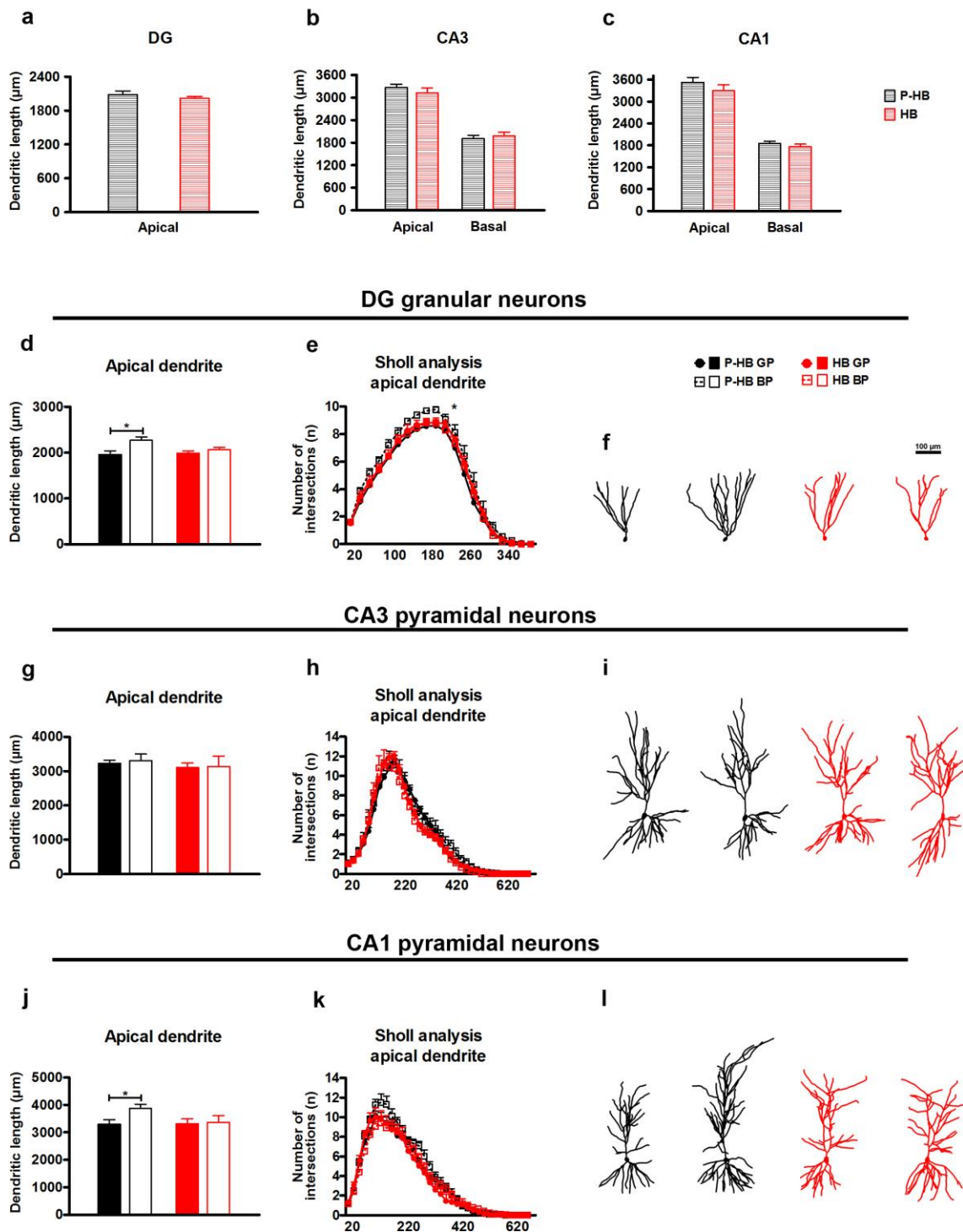
Figure 2| Morphological analysis of dorsal HPC neuron dendritic arborizations. A, B and **C)** Comparison of dendritic lengths of DG granular, CA3 and CA1 pyramidal neurons between the P-HB and HB group. **D,G** and **J)** Average apical dendritic lengths of DG granular **(D)**, CA3 **(G)** and CA1 **(J)** pyramidal neurons for the P-HB and HB group, both GPs and BPs. For each group, GPs and BPs clustering were determined based on the reference memory performance. **E, H** and **K)** Sholl analysis of the apical dendrite of DG granular neurons **(E)**, CA3 **(H)** and CA1 **(K)** pyramidal neurons for the P-HB

and HB group, both GPs and BPs. This graph presents the mean number of intersections of apical dendritic branches with consecutive 20µm spaced concentric spheres. **F, I** and **L)** Representative reconstructions of DG granular neurons **(F)**, CA3 **(I)** and CA1 **(L)** pyramidal neurons used in the previous analysis. Number of animals: 15 Pseudo HB animals (GPs=9, BPs=6) and 13 HB animals (GPs=5, BPs=8). Error bars represent SEM; *p< 0.05 - GPs vs BPs only in P-HB group.

Since our previous work (Mota *et al.*, 2018) has shown that aged control animals can be clustered as GPs and BPs with characteristic age-related dendritic morphologies, we assessed whether cluster membership for RM performance could unmask an underlying effect of HB cognitive training on dendritic tree plasticity. Thus, Pseudo-HB and HB animals were clustered by a k-means cluster analysis according to RM performance as assessed in the water maze. A two-way ANOVA revealed no significant effects of cognitive training or performance on mean apical dendritic length of DG granular, CA3 and CA1 pyramidal neurons (Table 1 and Fig. 2d,g,j). However, an interaction between these factors was observed for granular neurons (Table 1 and Fig. 2d). Data for basal dendrites of CA3 and CA1 pyramidal neurons revealed no significant effect of training or performance, neither an interaction between them (Table 1). Within group comparisons showed that Pseudo-HB BPs had longer apical dendritic lengths, both for DG granular neurons and CA1 pyramidal neurons, when compared with Pseudo-HB GPs (Granular neurons: t(13)=-2.803, p=0.015, d=1.520; CA1 apical dendrite: t(13)=-2.475, p=0.0.028, d=1.340) (Fig 2d,j). No differences were found between CA3 apical dendrites of both Pseudo-HB GPs and BPs (Fig. 2g). Regarding the HB group, no significant differences were found between GPs and BPs, both for DG granular neurons, CA3 and CA1 pyramidal neurons (Fig 2d,g,j). No differences were also reported regarding basal CA3 and CA1 pyramidal neuron dendrites for both Pseudo-HB and HB training groups (Data not shown). Sholl analysis of the distribution of apical dendritic material, for DG, CA3 and CA1 neurons, as a function of distance from the soma did not reveal any significant effect of cognitive training or performance (Table 1 and Fig. 2e,h,k). However, an interaction between these two factors was observed solely for granular neurons (Table 1 and Fig. 2e). Additionally, within group comparisons showed a significant increase of Pseudo-HB BPs dendritic material ($F_{(1,13)}$=7.395, p=0.018, $\eta\rho^2$=0.363) at distances between 40-200µm when compared with Pseudo-HB GPs. These alterations are exemplified in the reconstruction samples of figures 2f,i,l.

4.3.2. Cognitive training prevents hippocampal volumetric metrics characteristic of old age bad performing animals

The effects of cognitive training at 10 months on HPC volume were also analyzed by a detailed study performed on the DHPC (Fig. 3). When we analyzed the entire extent of the DHPC, no differences were found between Pseudo-HB and HB groups ($t(21)=-1.929$, $p=0.067$, $d=0.803$) (Fig. 3a).
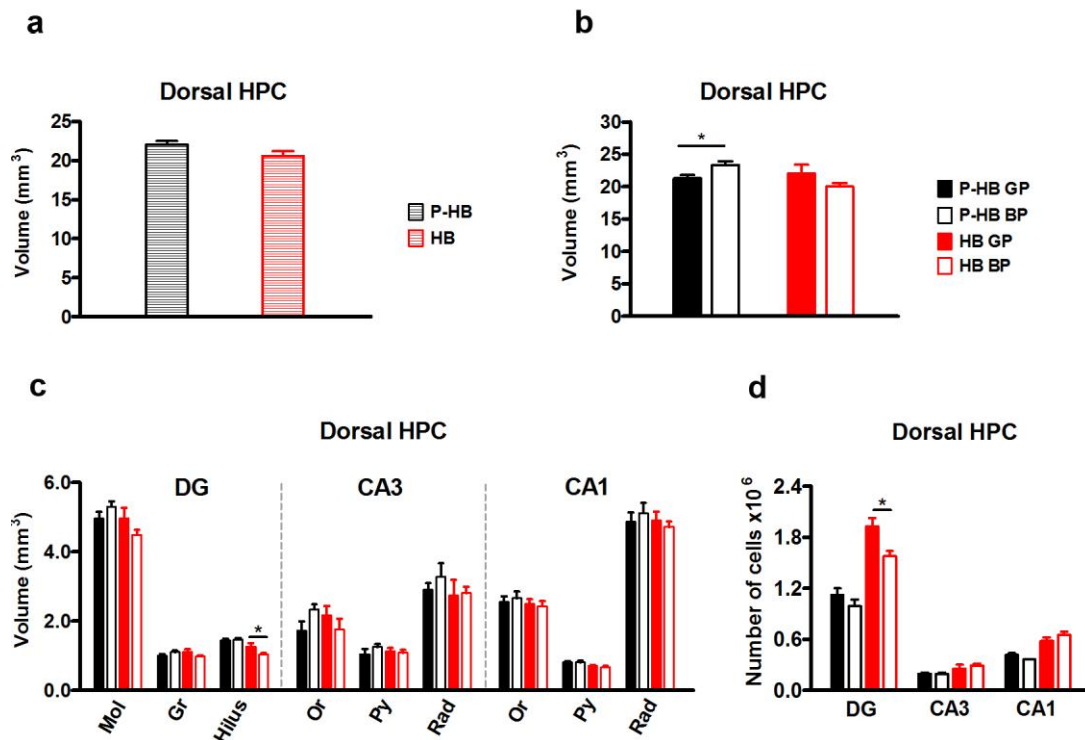
Volumetric analysis

Figure 3| Cognitive training prevents volumetric alterations in old age. A) Volume of the dorsal hippocampal formation for the P-HB and HB group. **B)** Average volumes of the dorsal hippocampal formation for the P-HB and HB group, GPs and BPs. GPs and BPs clustering were determined based on the reference memory performance. P-HB BP have a higher DHPC volume when compared with the GPs. **C)** Analysis of the volumes of different subregions of the dorsal hippocampus. **D)** Estimated number of neurons in granular layer of dentate gyrus and pyramidal layer of CA3 and CA1 for DHPC. Number of animals: 14 Pseudo HB animals (GPs=9-8, BPs=5) and 11 HB animals (GPs=3-4, BPs=7). Error bars represent SEM; *p<0.5.

Therefore, as previously performed for the dendritic morphology assessments, we next performed a k-means clustering based on water maze RM performance (Fig. 3b). Two-way ANOVA revealed no significant effect of training or performance on the volume of DHPC, with a significant interaction between them (Table 1 and Fig. 3b). Within group comparisons, in accordance with the already presented data on

dendritic lengths, showed that Pseudo-HB GPs presented a decreased DHPC volume when compared with the BPs animal group ($t(11)=-2.530$, $p=0.028$, $d=1.442$) (Fig. 3b). Likewise, no differences between HB GPs and BPs were found (Fig. 3b) We next analyzed the various subregions of the DHPC, namely: the DG (molecular, granular layer and hilus), the CA1 and the CA3 (strata oriens, pyramidal and radiatum). A two-way ANOVA revealed a significant effect of cognitive training in the volume of the hilus and the pyramidal layer of CA1 (Table 1 and Fig. 3c). In all studied regions, no significant effect of performance was observed (Table 1 and Fig. 3c). An interaction between these two factors was reported solely for the granular layer of DG (Table 1 and Fig. 3c). Within group comparisons showed no significant differences between Pseudo-HB GPs and BPs, while for the HB training group, a significant difference was reported regarding hilus volume, with BPs presenting a decreased volume when compared with GPs ($t(8)=2.581$, $p=0.033$, $d=1.516$) (Fig. 3c).

4.3.3. Increased hippocampal neuron number after cognitive training in adulthood

A two-way ANOVA revealed a significant effect of cognitive training at 10 months on the number of DHPC dentate granule, CA3 and CA1 pyramidal cells (Table 1 and Fig. 3d). A significant effect of performance was observed only for DHPC granular layer neurons (Table 1 and Fig. 4d). No significant interaction between training and performance was found (Table 1 and Fig. 3d). Within group comparisons revealed no differences among Pseudo-HB GPs and BPs (Fig. 3d). Nevertheless, concerning the HB group, a significant difference was observed in the number of DHPC granular layer cells, with GPs presenting an increased number of neurons when compared with BPs animals ($t(8)=2.963$, $p=0.018$, $d=2.009$) (Fig. 3d).

5. Discussion

Nowadays, cognitive training has been one of the strategies used to achieve successful cognitive aging (Willis *et al.*, 2006; Nouchi *et al.*, 2012; Jiang *et al.*, 2016). Since the HPC is particularly vulnerable to aging (Mora *et al.*, 2007), in this study we used the HB test as a cognitive training task (1-week protocol) (Van der Staay, 1999; Depoortère *et al.*, 2010).

The HB task is a maze-like test of spatial learning and memory (Van der Staay, 1999; Depoortère *et al.*, 2010). This task relies on the same concept as the Morris water maze, i.e, having an open-field design, usually square, that provides distal cues while being uniform within the apparatus to prevent use of proximal cues (Vorhees & Williams, 2014). By using distal orientation, with no local cues to guide

behavior, the animal must learn the spatial location of the goal object relative to several distal cues (Morris, 1981). In our study, by assessing spatial learning across repeated unchanged trials, we were aiming to engage trial-independent memory performance, thus training spatial reference memory, with minor engagement of working memory skills (Van der Staay *et al.*, 2012).

Our work revealed that exposure to cognitive training in adulthood (10 months of age) prevented reference memory impairments later on. By contrast, no changes in working memory and behavioral flexibility skills were observed. These observations strengthen the view that training on specific "cognitive tasks" may serve to reinforce or reactivate circuits and therefore to (at least partially) restore some of the age-induced deficits in cognition. In agreement with this data, Harburger *et al.*, 2007 reported that all enrichment treatments, including cognitive stimulation, improved spatial memory in aged females, indicating that either exercise or cognitive stimulation can improve memory in aged subjects. Interestingly, our work showed that the effects of a short period of cognitive training (1-week protocol) are long lasting in time. This points out that engagement in a highly cognitive demanding task, even for a relatively short period of time, is able to enhance cognitive function. In accordance, Arai *et al.*, 2009 demonstrated that when 15-day old mice were subjected to 2 weeks of EE they exhibited enhanced hippocampal LTP. Later, when the same mice were breed and LTP was analyzed in their offspring, now reared in standard cages, hippocampal LTP was enhanced when compared to offsprings from non-enriched parents. Similarly, Cheng *et al.*, 2012 showed that in old adults, the effects of interventions on cognition are maintained 1 year after the training has ended.

Taken together, these findings could be explained by the cognitive reserve hypothesis, which rests on the ability of the brain to compensate for pathological changes associated with aging, depending on the previous stage of intellectual capability (Whalley *et al.*, 2004). This means that people with greater cognitive reserve can tolerate more the neurodegenerative brain changes associated with dementia or other brain pathologies, such as Parkinson's disease, multiple sclerosis, or stroke (Stern, 2012). Likewise, in our work, it seems that aged rats with higher cognitive reserve, as a consequence of the exposure to the HB test, cope better with age-related deterioration than the aged counterparts with lower cognitive reserve. Therefore, an important goal of aging research should be the promotion and sustainment of elderly people cognitive reserves.

Furthermore, our findings highlight the importance of the age at which individuals are exposed to cognitive stimulation (reviewed by Frick, 2010). The influence of age to the exposure to EE seems to be controversial. Most studies indicate that EE at young ages appears to have even more beneficial effects which is most likely due to the fact that brain development is not complete and more-long lasting

130

molecular and anatomical changes are induced (Bouet *et al.*, 2011; Freret *et al.*, 2012). However, other studies found positive effects when EE training was initiated in already aged and cognitively impaired rodents (Bennett *et al.*, 2006). In accordance with this data, we also reported that 10 months of age seems to be the best sensitive period for the effectiveness of cognitive training, however we did not test animals younger than this time point.

Our goal was also to address the underlying mechanisms of cognitive training. It is known that cognitive stimulation induces structural and functional alterations in the brain (Kolb *et al.*, 2003; Bindu *et al.*, 2007; Artola *et al.*, 2006, Freret *et al.*, 2012). Accordingly, a large body of literature has shown that exposure to an enriched environment enhances dendritic branching in rats (Greenough & Volkmar *et al.*, 1973; Green *et al.*; 1983; Kolb *et al.*, 2003; Bindu *et al.*, 2007), as well as in humans (Jacobs *et al.*, 1993). However, some studies failed to see such differences (Diamond *et al.*, 1976), while others reported differences in dendritic branching in dentate granule cells, but only in female EE rats (Juraska *et al.*, 1985, 1989). Regarding volumetric changes, an increase in cortical thickness in both rats (Mohammed *et al.*, 2002) and healthy elderly individuals (Jiang *et al.*, 2016; Seider *et al.*, 2016) was seen after cognitive training, while another study also reported that aerobic exercise reversed age-related decreases in hippocampal volume, which correlated with an improvement in spatial skill (Erickson *et al.*, 2011).

In the present work, structural analysis confirmed a change in neuronal length and HPC volume of worse HB performing animals that reached the level of the best performing group. Also, no significant effects of training were reported for the dendritic length or HPC volume. These findings are in contradiction with the majority of studies using EE animal models and cognitive training in humans, which report that exposure to an enriched environment enhances dendritic branching (Greenough & Volkmar *et al.*, 1973; Green *et al.*, 1983; Kolb *et al.*, 2003; Bindu *et al.*, 2007; Jacobs *et al.*, 1993). Of note, most of these structural studies were performed in adult rats which made it difficult to compare with our own data. Nevertheless, our results are in accordance with our previous data showing that in aged individuals "smaller is better" (Mota *et al.*, 2018), as the cognitively trained BP group reached the level of the best performing animals. Accordingly, aged GP untrained individuals had smaller DG and CA1 dendritic trees and smaller HPC volume when compared to BP untrained animals.

Further adding to these findings, an increased HPC neuron number was observed in the cognitively-trained group. This finding could imply that, in these animals, the aged brain is supplemented with more neurons simply as a result of new learning. Indeed, several studies reported EE induces neurogenesis (Greenough & Volkmar, 1973; Faherty *et al.*, 2003; Rampon *et al.*, 2000). Also, it´s known that HPC neurogenesis is strongly improved by physical activity, especially aerobic exercise (van Praag *et al.*, 1999;

131

Steiner *et al.*, 2008). In contrast, mental training via skill learning increases the number of surviving neurons, particularly when the training goals are challenging (Gould *et al.*, 1999; Shors; et al., 2012; Wurm *et al.*, 2007). Therefore, and bearing in mind that future work should specifically tackle this question, we hypothesize that an increase in cell survival should be the principal mechanism driving the increased neuron number.

In conclusion, our work suggests that the HB test is a useful tool to enhance cognitive function, and its effects are circuitry-specific and long lasting in time. The beneficial effects of cognitive training are potentially mediated by alterations in dendritic branching. These promising findings highlight the existence of continuous functional plasticity, which brings optimism about the possibility of promoting "mindspan".

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Table 1| Results of two-way ANOVA on the data from Pseudo-HB and HB animals.

	df	Training (HB VS P-HB)			Reference memory performance			Interaction		
	df	F	P	ηρ²	F	P	ηρ²	F	P	ηρ²
Hippocampal formation										
Morphology of neurons										
Granular neurons	1,24	1.730	0.201	0.067	2.893	0.102	0.108	**7.751**	**0.010**	**0.244**
CA3 pyramidal neurons (apical dendrite)	1,24	0.822	0.374	0.033	0.022	0.882	0.001	0.095	0.760	0.004
CA3 pyramidal neurons (basal dendrites)	1,24	0.037	0.849	0.002	3.276	0.083	0.120	0.020	0.889	0.001
CA1 pyramidal neurons (apical dendrite)	1,24	2.132	0.157	0.082	2.410	0.134	0.091	1.942	0.176	0.075
CA1 pyramidal neurons (basal dendrites)	1,24	1.288	0.268	0.051	0.886	0.356	0.036	0.006	0.939	0.000
Sholl analysis										
Granular neurons	1,24	0.845	0.367	0.034	3.132	0.089	0.115	**6.839**	**0.015**	**0.222**
CA3 pyramidal neurons (apical dendrite)	1,24	0.782	0.385	0.032	0.001	0.972	0.000	0.007	0.936	0.000
CA1 pyramidal neurons (apical dendrite)	1,24	3.100	0.091	0.114	1.943	0.176	0.075	0.992	0.329	0.040
Volumetric measurements										
Dorsal HPC	1,19	3.367	0.082	0.151	0.000	0.999	0.000	**8.291**	**0.010**	**0.304**
Dorsal – DG molecular layer	1,19	3.769	0.067	0.166	0.125	0.727	0.007	3.867	0.064	0.169
Dorsal – DG granular layer	1,19	0.010	0.920	0.001	0.130	0.723	0.007	**5.680**	**0.028**	**0.230**
Dorsal – DG hilus	1,19	**25.639**	**<0.0005**	**0.574**	2.563	0.126	0.119	4.168	0.055	0.180
Dorsal – CA3 stratum oriens	1,19	0.049	0.827	0.003	0.128	0.724	0.007	2.828	0.109	0.130
Dorsal – CA3 pyramidal layer	1,19	0.052	0.822	0.003	0.307	0.586	0.016	0.836	0.372	0.042
Dorsal – CA3 stratum radiatum	1,19	1.192	0.289	0.059	0.616	0.442	0.031	0.279	0.603	0.014
Dorsal – CA1 stratum oriens	1,20	0.683	0.418	0.033	0.021	0.886	0.001	0.234	0.634	0.012
Dorsal – CA1 pyramidal layer	1,20	**8.435**	**0.009**	**0.297**	0.125	0.728	0.006	0.245	0.626	0.012
Dorsal – CA1 stratum radiatum	1,20	0.465	0.503	0.023	0.016	0.899	0.001	0.653	0.428	0.032
Total number of neurons										
Dorsal										
DG granular layer	1,19	**63.035**	**<0.0005**	**0.768**	**7.528**	**0.013**	**0.284**	1.512	0.234	0.074
CA3 stratum pyramidal	1,20	**10.864**	**0.004**	**0.352**	0.489	0.492	0.024	0.591	0.451	0.029
CA1 stratum pyramidal	1,20	**40.583**	**<0.0005**	**0.670**	0.050	0.825	0.002	2.989	0.099	0.130

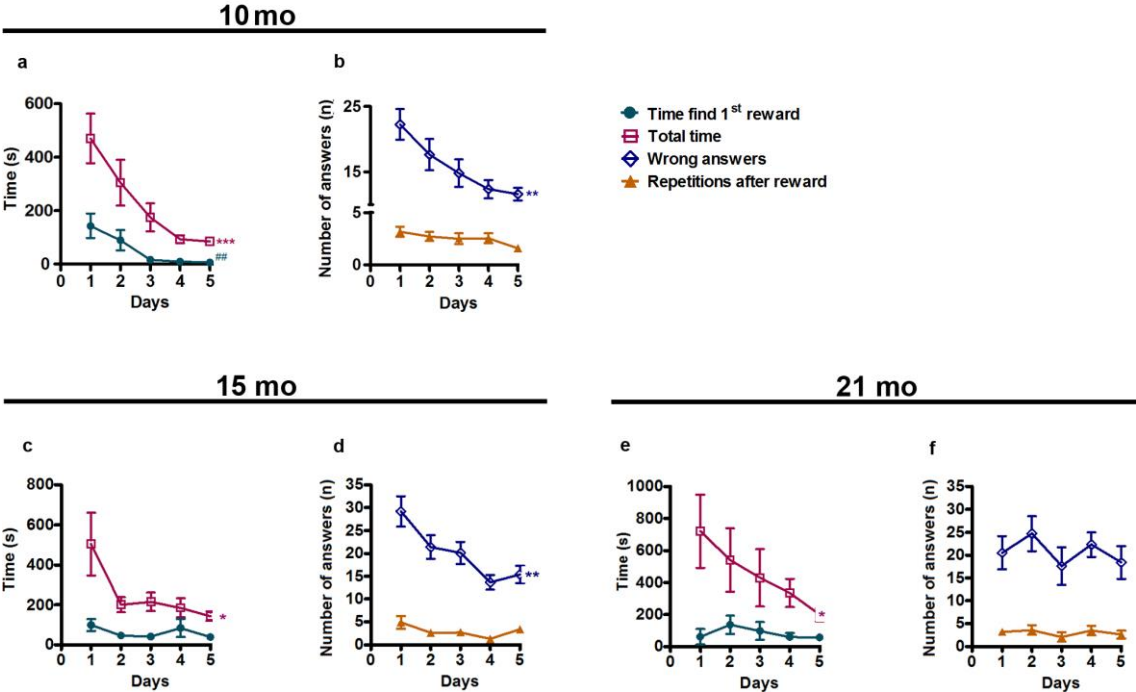## Cognitive training - Hole Board task

Figure 1| Analysis of the performance on the Hole board task. Hole Board performance in animals with: **A, B)** 10 months of age (n=22); **C, D)** 15 months of age (n=9); **E, F)** 20 months of age (n=11). **A, C, E)** Time to find the first reward and total time to finish the task. **B, D, F)** Number of repetitions after reward (working memory errors) and number of wrong answers (reference memory errors). Error bars represent ± SEM; *p<0.05, **p<0.01. (mo: months).

Cognitive assessment
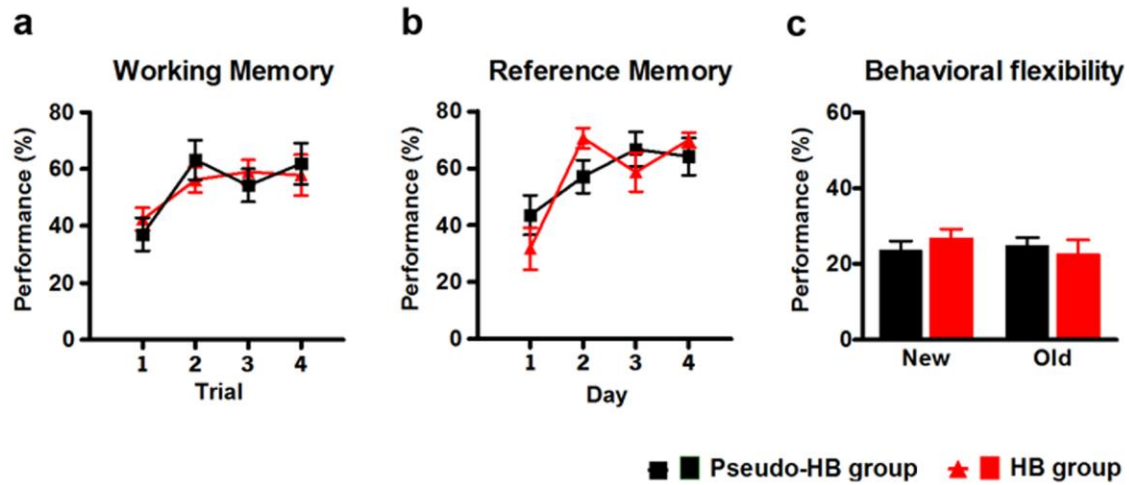
15 mo
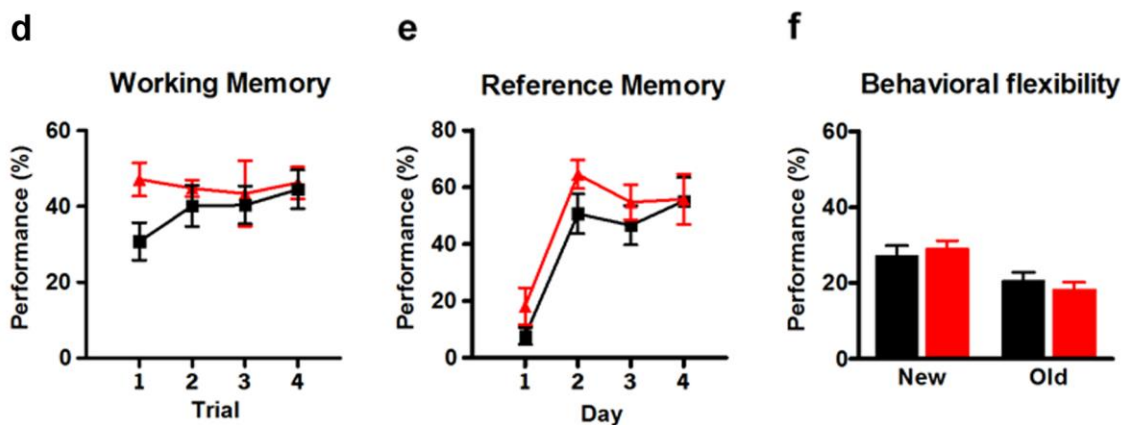
Figure 2| No impact of cognitive training performed in 15 and 20 month-old animals.
Learning curves in the working **(A,D)** and reference memory tasks **(B,E)** of Pseudo-HB and HB groups.
No differences between groups were found. **C,F)** Results from the behavioral flexibility task. Average time
spent on the four trials in the new and old quadrants is given as a percentage of the total escape latency.
No differences between groups were found. Number of animals: 15 months group, Pseudo-HB n= 9, HB
n=11; 20 months group, Pseudo-HB n=9, HB n=11; 10 months group, Error bars represent ± SEM.

General discussion, conclusions and future perspectives

The present work showed that individual differences in cognitive aging can be ascribed, at least in part, to individual variations in dendritic pruning. Additionally, we also showed that training in a reference memory task during adulthood can have a long-lasting positive impact in cognitive aging, preventing age-associated reference memory deficits 1 year later. By revealing some of the biological basis for the complexities of brain-aging, the present work is an important step towards understanding age-related cognitive disorders, contributing to the development of new therapeutic avenues to prevent or restore cognitive impairment.

1. Experimental considerations:

1.1. Animal models

The primary aim of this thesis was to thoroughly characterize the cognitive function and its heterogeneity in the context of the normal aging process.

Aging studies present unique considerations due to their long duration and the significant amount of resources required. Therefore, there is an ongoing discussion regarding what represents the best model system to study age-related cognitive impairment. Different model systems, from *Caenorhabditis elegans,* to rodents, to nonhuman primates, or even to models of computational neuronal modeling, have been used to assess different aspects of cognitive impairment (Mitchell *et al.*, 2015). While each model presents inherent advantages and disadvantages, they all share a superior ability for controlling nearly uncontrollable variables among human individuals, as well as avoiding major ethical concerns normally faced when dealing with human trials (Granholm, 2010). Nonetheless, due to the complexity of the systems involved in cognitive processing, the study of age-related cognitive impairments does not lend itself well to invertebrate models or computational neuronal modelling; rather, rats have been extensively used to model fundamental aspects of human memory.

Rats provide an excellent and reproducible system to study aged-related cognitive decline. As already discussed, they present significant homologies with humans in the organization and function of brain systems (Bizon & Nicolle, 2006). For instance, age-related deficits tied to the function and structure of the HPC and mPFC are observed across rat strains and resemble prominent features of neurocognitive aging in humans (Driscoll *et al.*, 2003, 2006; de Brabander *et al.*, 1998; Markham & Juraska, 2002; Dickstein *et al.*, 2013). Particularly, similarly to humans, rats present a broad profile of both severity and nature of cognitive deficits (Ménard & Quirion, 2012).

Another major factor favouring the use of rodent models is their short lifespan. While humans live for around eight decades and do not manifest significant rates of memory decline until their fifth or sixth decade (Nyberg *et al.*, 2012), the lifespan of rats can be extended up to 30 months and they exhibit memory loss at 18–20 months of age with a significant decline at 24 months of age (Bizon & Nicolle, 2006; LaSarge & Nicolle, 2009). Equivalent ages between rodent and human have been defined by comparing survival rates between rats and humans over their lifespans (see table 3) (Sengupta, 2013). Thus, an aged animal with 24 months of age is equivalent to a 60 years old person (see table 3) (Sengupta, 2013). Hence, these animals are usually allowed to age naturally without the use of transgenic or knockout paradigms, because while the former mimics the natural aging process commonly found in human populations, the later could be affected by intrinsic developmental abnormalities and other confounders (Bizon & Nicolle, 2006; Mitchell *et al.*, 2015). Naturally, it is thus easier to perform longitudinal or cross-sequential studies of memory decline in rats than in humans. Accordingly, for the purpose of this study, we chose to use male Wistar Han rats allowed to age until 22-24 months old. As an outbred strain, Wistar Han rats present more genetic variation than inbred strains, which make them a good candidate to mimic the individual variability observed in aged human populations (LaSarge & Nicolle, 2009).

Our study was solely focused in male subjects. As previously shown, gender is an important variable to be considered when studying aged individuals (Hyde & McKinley, 1997; Gur & Gur, 2002; Sprott, 2011). Particularly, a large body of literature has shown the effects of sexual hormones (especially estrogens) on cognitive processes in both humans and rats (Luine, 2014). Therefore, we chose to focus our work on male rats since they provided a biological scenario free from the oestrous cycle. Even though, it is important to bear in mind that important sex differences can be noted in cognitive performance when comparing males and females (Bimonte-Nelson *et al.*, 2008).

In summary, the choice of the animal model is one of the most critical aspects of experimental design. For many reasons discussed above, rats have been extensively used as a laboratory animal model of aging in behavioral studies of cognitive processing. Despite of this, it is important to be aware that rats are not a miniature form of humans; differences in anatomy, physiology, development and biological phenomena must be taken into consideration when analyzing the results of any research performed in this animal model. Hence, translation from rodents into successful outcomes for humans must be carefully analyzed.

Table 3: Rat´s age in human years (adapted from Sengupta, 2013).

Rat age (years)	Human age (years)
6 months (0.5)	18
12 months (1.0)	30
18 months (1.5)	45
24 months (2.0)	60
30 months (2.5)	75
36 months (3.0)	90
42 months (3.5)	105
45 months (3.75)	113
48 months (4.0)	120

1.2. Behavioral paradigms

1.2.1. Water maze-based tests

Rats cannot describe their experiences directly, making the investigation of certain cognitive functions, such as episodic memory, difficult. Therefore, the range of memory functions studied in the rat model is mostly limited to spatial learning and fear conditioning. Thereby, to make inferences about the aging process from the animal model back to humans, rigorous behavioral paradigms must be used to ensure that the same function is being examined across species. Fortunately, the domain of spatial memory provides a common ground between species and happens to be a domain where age-related deficits are described consistently for humans and rats (Gallagher & Rapp, 1997). Accordingly, we used a battery of water maze tests to thoroughly characterize learning and memory processes related to the ability for spatial orientation.

In 1984 Morris described for the first time the MWM task, a method to assess spatial or place learning (Morris, 1984). Several modifications of this task were later designed to assess other forms of learning and memory (Stewart & Morris, 1993; Kesner, 2000). These water maze-based tests exploit the natural and remarkable ability of rats to swim; however, rats still prefer to be out of water, and thus swimming provides sufficient motivation for animals to actively search for an escape (Vorhees & Williams, 2006). Furthermore, these water maze tests have several advantages over traditional mazes. They are easy to perform, little training is required, they eliminate the need for food deprivation, as getting into the platform is a reward in itself, they are insensitive to differences in body weight and appetite, they have cross-

species utility [rats, mice and humans - in a virtual maze (Kallai *et al.*, 2005)] and they avoid the problems of dry mazes with respect to olfactory guidance (e.g. by urine marks) and other intramaze cues (Vorhees &Williams, 2006).

Another advantage of water maze tests is the ability to control for motor ability which could impact on task performance for reasons not directly related to spatial learning (Gallagher *et al.*, 1993). Thus, in water maze tests, escape from water is relatively immune from activity or body mass differences, making it ideal for many experimental models (Vorhees & Williams, 2006). Also, by assessing swim speed during learning, one can determine whether animals have impaired ability or motivation to escape (Fitzgerald & Dokla, 1989). Nevertheless, these tests are not immune to other confounders such as altered emotionality, as animals can adopt passive (i.e. extensive floating) or active coping strategies (escape behavior along the walls) (Wotjak, 2004; Sousa *et al.*, 2006). However, special attention was given to identify the presence of animals with a tendency towards thigmotaxis (Vorhees & Williams, 2006), and whenever these behaviors were observed, animals were excluded from the test.

Lastly, one of the major limitations of the water maze test in aging research includes the problematic of the test-retest effect, making interventional or longitudinal studies difficult to conduct (LaSarge & Nicolle, 2009). Therefore, to avoid this possible complication, we designed a cross-sectional study where animals were tested only once in water maze tasks. Also, due to this possible confounding factor, a non-water maze task was selected for animal cognitive training.

1.2.2. Cognitive training - HB task

The HB task is a maze-like test of spatial learning and memory (Van der Staay, 1999; Depoortère *et al.*, 2010). This task relys on the same concept as the MWM, i.e, having an open-field design, usually square, that provides distal cues while being uniform within the apparatus to prevent use of proximal cues (Vorhees & Williams, 2014). By using distal orientation, with no local cues to guide behavior, the animal must learn the spatial location of the goal object relative to a number of distal cues (Morris, 1981). In our study, by assessing spatial learning across repeated unchanged trials, we were aiming to engage trial-independent memory performance, thus training spatial reference memory, with minor engagement of working memory skills (Van der Staay *et al.*, 2012).

Therefore, and bearing in mind that a specific cognitive stimulation could differently and specifically enhance characteristic brain circuits, the HB food-retrieval task was used as a cognitive-enrichment tool for spatial reference memory training (hippocampal-dependent task) (Van der Staay, 1999; Depoortère *et al.*, 2010).

146

1.3. Structural Analysis

1.3.1. 3D-reconstruction of Golgi-cox impregnated neurons

One of the most commonly used techniques to evaluate the morphology of nerve cells is the Golgi impregnation technique, initially termed 'the black reaction' (Golgi, 1873; Cajal, 1952). Hence, in our work, we performed the Golgi-cox staining according to the protocol of Gibb & Kolb, 1998. This is one of several heavy metal impregnating methods (in this case mercury) derived from the original silver impregnation technique developed by Camilo Golgi (Golgi, 1873). One of the main strengths of the Golgi method is that it stains only approximately 1-10% of neurons in any one selected region (Shankaranarayana *et al.*, 2004), allowing a panoramic visualization of virtually all parts of an animal neuron. Thereby, using this procedure, the dendrites and spines are evenly and consistently stained for brains of rats ranging in age from postnatal day 0 until old age (Gibb & Kolb, 1998). Other advantages include the ability to stain and analyse relatively thick sections (200um), thus allowing the 3D reconstruction of an entire dendritic tree, as well its low technical difficulty and extremely affordable cost. Because of these characteristics, the Golgi stain is still the gold standard for analysis of dendritic arborizations. However, there are a number of recent techniques to obtain precise reconstructions of the dendritic arborizations. These techniques include selective intracellular injection of fluorescent dyes (Radley *et al.*, 2004) or the use of transgenic mice expressing GFP in a subpopulation of neurons (Bas Orth *et al.*, 2005). Nevertheless, the golgi technique remains a cost-effective and easy to implement technique, thus remaining a widely used method for whole neuron visualization and elegant detailed analysis of dendritic arborization and dendritic spine phenotypes (Gibb & Kolb, 1998).

1.3.2. Stereological procedures

Stereological determinations of neuronal numbers and volumes of a region of the brain provide an important perspective of its general structure. In this sense, stereology refers to the group of techniques used to accurately estimate quantifiable characteristics of 3D objects, such as volumes or number of particles. The use of these tools allows a precise estimate of the numeric value of a parameter without actually having to measure it (Gundersen *et al.*, 1999).

In the present work, volumes and neuronal numbers in the HPC and mPFC were assessed using unbiased stereological tools (Cavalieri principle and optical fractionator, respectively) in Giemsa-stained methacrylate-embedded brain sections (Gundersen *et al.*, 1988; West *et al.*, 1991). A major constraint of this technique relies on the fact that it represents a steady snapshot at the time of sacrifice. One of the

possibilities to overcome this shortcoming could be the use of *in vivo* imaging techniques (eg. magnetic resonance images) (Kalisch *et al.*, 2004). Nevertheless, these imaging techniques remain relatively expensive to use, when compared to the classical methods we employed, and would have a maximal advantage in a longitudinal type of study, unlike the cross-sectional study we designed.

1.4. Molecular analysis

Autophagic activity has been identified as a critical mechanism underlying dendritic remodeling (Bingol & Sheng, 2011). To analyze the levels of autophagic activity in the HPC and mPFC, we performed western blot analysis of the autophagosome markers LC3 and p62. In case of decreased autophagic activity, the level of LC3-II (lipidated LC3), a biomarker that indicates the abundance of autophagosomes, is decreased, while the level of autophagy substrates p62 is increased (Tang *et al.*, 2014). To determine the relationship between autophagic and neurotrophic activity, protein levels of brain-derived neurotrophic factor (BDNF) were analyzed. BDNF is one of the members of the neurotrophic factors family and is a main inducer of dendritic and spine growth (Tanaka *et al.*, 2008). Thus, higher levels of this neurotrophic factor suggest an increase in dendritic growth. Also, given the technical challenge to assess protein levels and dendritic tree length in the same region of the same animals, these results were correlated with pre-[synaptophysin (SYP) and synaptosomal-associated protein 25 (SNAP25)] and post-synaptic [postsynaptic density protein 95 (PSD95)] markers. Indeed, there is a consensus in the literature that the levels of these markers, particularly when concordant, are a good surrogate of synaptic abundance and dendritic tree complexity (Fletcher *et al.*, 1991; Marrs *et al.*, 2001; Tomasoni *et al.*, 2013).

2. Impact of normal aging in cognitive performance and its inter/intra-individual differences

As already stated in the introduction (chapter I section 4.1), normal aging corresponds to the inevitable natural changes that aged individuals experience in their biological systems, that are not necessarily harmful to the individual (Harada *et al.*, 2013). Thereby, as longevity increases, different structural and functional alterations in brain architecture have been observed, which may culminate in learning and memory impairments (Glisky, 2007). Even excluding pathological aging (dementia and other illnesses associated with age), age-related changes in human cognitive functions differ in extent and detail from individual to individual, with some individuals aging more successfully than others (Ardila *et al.*, 2003; Harada *et al.*, 2013; Santos *et al.*, 2013).

In an attempt to clarify the observed heterogeneity in the normal aging brain, we performed a detailed behavioral analysis of the cognitive function of both young and old rats (Chapter II). It is known that rats exhibit morphological, biochemical, and metabolic changes in their brains, as well as cognitive deficits, with aging (Gage *et al.*, 1988). A standard approach used to model the deficits associated with aging, consists in allowing rats to age naturally until the desired period which better mimics the normal aging process occurring in humans (LaSarge & Nicolle, 2009; Mitchell *et al.*, 2015). Consistent with previous studies, the data herein presented confirmed that old rats, as a group, showed poorer spatial learning and behavioral flexibility than young subjects (Rapp & Gallagher, 1996; Syková *et al.*, 2002; Ménard & Quirion, 2012; McQuail & Nicolle, 2015). Importantly, the performance of both young adult and older rats in working and reference memory tasks was highly heterogeneous, particularly in older individuals; in the latter, we confirmed that a certain proportion of subjects maintained spatial memory abilities comparable to those of younger animals (Ménard & Quirion, 2012; McQuail & Nicolle, 2015), which indicates that cognitive aging is not inevitable or strictly linked to chronological age. Of notice, the data clearly revealed, for the first time, that this age-associated increase in the dispersion of individual performance is not universal, as it was not present in all cognitive dimensions (e.g. the behavioral flexibility task). Hence, the present work clearly showed that old rats display a broad range of cognitive decline which mimics the heterogeneity observed in the human population (Ardila *et al.*, 2003; Harada *et al.*, 2013; Santos *et al.*, 2013). This similarity makes rats a good model to study the normal aging process and allow us to further address the basic mechanism underlying the inter/intra-individual differences.

Beyond individual differences, aging also influences certain cognitive domains and types of memory more than others. For example, in humans, the cognitive domains most affected by normal aging and the most susceptible to brain damage are associated with episodic and spatial memory, while other cognitive processes are relatively unaffected (namely verbal skills, implicit and semantic memory) (Raz, 2000; Glisky, 2007; Nyberg *et al.*, 2012; Fjell *et al.*, 2014).

The working and reference memory tasks performed in this work both assess spatial learning and memory, albeit within a different timeframe: while the former is dependent on short-term memory and HPC to mPFC connections (Goldman-Rakic, 1995; Kesner, 2000), the latter depends on long-term memory and largely on the integrity of the HPC (Morris, 1984). In contrast, the behavioral flexibility task is memory independent and assesses the ability to adapt to changing circumstances (de Bruin *et al.*, 1994), a critical component of executive function, which is a very distinct cognitive ability. Particularly in our work, this task reveals whether or not animals can extinguish their initial learning of the platform's position and acquire a new memory. In light of this distinction, the observation that aging is accompanied

by an increased inter-individual heterogeneity (and a performance decline, on average) in memory-dependent but not executive-function-dependent tasks adds yet another layer of heterogeneity to the aging process, and strongly suggests that some cognitive functions (and the networks sub-serving them) are more prone to aging than others. Significantly, this seems also to be the case in humans in which both long-term and working memory are more influenced by age-related impairments than knowledge of vocabulary and priming, a form of non-declarative memory (Buckner, 2004). Despite this, the herein reported relative preservation of executive function in rodents might seem to contradict several studies in humans showing that executive processes are also disrupted in aging (Schacter *et al.*, 1991; Johnson *et al.*, 1993; West, 1996). However, it is important to highlight that, besides the obvious species difference, executive function tasks in humans are often contaminated by deficits in speed of processing (Head *et al.*, 2009), which are well known to be affected by aging.

In addition to the two layers of heterogeneity in aging discussed above, and given the variability observed in the behavioral characterization, for each behavioral test, animals were classified as good and bad performers (GP, BP) according to their individual cognitive performance. Unlike previous studies (Robitsek *et al.*, 2008; Ménard & Quirion, 2012; McQuail & Nicolle, 2015), we categorized as GPs and BPs not only the old animals, but also the youngsters. Importantly, it was shown that the group of GPs and BPs were not the same for the three different tasks assessed. Thus, by using this approach, we were able to obtain more detailed information about the cognitive status of the animals, which further revealed another dimension of inter-individual heterogeneity. Indeed, when comparing cluster membership for each individual in each test, we showed for the first time that most animals were GPs in some tests and BPs in others, without a clear separating pattern in either younger or older groups. Curiously, individuals impaired in one task, were not necessarily the same individuals impaired in another task. It seems that cognitive changes across the brain are task-dependent, and, subsequently, region-specific.

More importantly, this third heterogeneity level seems to be independent of the other two. In other words, despite all the inter-individual heterogeneity (within a given test) and the heterogeneity between tests (with reference and working memory being more sensitive to aging than behavioral flexibility) there is also heterogeneity at the individual level, as to being a GP or BP for each behavioral test.

In summary, the behavioral data suggested that cognitive decline in aging is not inevitable, or strictly linked to chronological age and that even in a relatively homogeneous population of animals such as the one in this study, there is a high variability and complexity in the way the different cognitive functions are preserved/impaired in each individual. Understanding the underpinnings of individual differences may

help to explain the observed heterogeneity and, possibly, what determines the existence of healthier agers, which is next discussed at the structural and molecular levels.

3. Heterogeneity in cognitive aging correlates with structural changes in the HPC and mPFC

As already mentioned, one of the most striking characteristics of cognitive aging is its heterogeneity. Due to this compelling evidence and considering the role of the HPC and mPFC in cognitive function, using young and old rats, our aim was to uncover the structural correlates of such behavioral differences by performing 3-dimensional neuronal reconstructions and volumetric analysis in these two key regions.

As already discussed, a large body of literature has examined the state of neurons during aging, for both humans and rats, and has demonstrated contradictory results. Some studies reported an age-related regression in the dendritic arbors of the neuronal populations in the HPC and mPFC, while others showed the opposite or even an absence of dendritic remodelling (for more details see table 1 and 2 presented in chapter I). Regarding volumetric alterations, contradictory findings have also been reported. This general lack of agreement in findings could be ascribed to a generalized failure to take into account the heterogeneity observed in the aging brain.

In the present work, we performed 3D reconstructions of HPC and mPFC neurons, and we showed that, on average, older animals have shorter apical dendritic arborizations in dorsal HPC neurons (dentate gyrus granules and CA1 and CA3 pyramids) but similar apical dendritic trees in mPFC neurons (Cg/PL and IL layers II/III pyramids) when compared to younger animals. These findings are in line with most previous studies that analysed one or the other region (HPC: Geinisman *et al.*, 1978; Machado-Salas & Scheibel, 1979; Lolova *et al.*, 1989; Luebke & Rosene, 2003; Markham *et al.*, 2005; Chen *et al.*, 2014; mPFC: Anderson *et al.*, 2014; Allard *et al.*, 2012; Kougias *et al.*, 2016) and might suggest that the frontal regions might be less affected by the aging process or that age-related changes in neuronal morphology appear later in the mPFC. This is partly corroborated by the fact that age-related apical dendritic retraction in the mPFC was only reported in one study, when comparing animals with 28 months with subjects with 18, 8 and 2 months (Grill & Riddle, 2002). Interestingly, this relative mPFC ''resilience'' might be specific for the superficial layers, since deeper, layer V pyramidal neurons, similar to HPC cells, exhibit age-related apical dendritic retraction at 20-22 months (Markham & Juraska, 2002; Chen *et al.*, 2014; Kougias *et al.*, 2016). Importantly, this has been observed in humans, in which age-related dendritic retraction, in the same individuals, was 3 times more prominent in the deep than in the superficial PFC pyramids

151

(Nakamura *et al.*, 1985). The fact that mPFC dendrites are less affected by aging fits perfectly with the behavioral data presented here, pointing to an attenuated age-associated decline of executive functions. Another major novelty of the present work is the finding that older animals with deficits in HPC-dependent tasks have larger dendritic trees in the HPC than cognitively intact rats of the same age. Indeed, while for younger animals the apical dendritic trees of DG and CA1 neurons presented a significant positive correlation with the individual performances in the reference memory task, in older animals this correlation was reversed, with better performers having the smallest apical trees in all three regions analysed (DG, CA3, CA1). This association in older animals, between larger dendritic trees and poorer cognitive function, might be considered contra-intuitive. Of note, a previous study had already shown an increased basal dendritic length and complexity in CA1 hippocampal cells of male rats with aging, but these animals were not cognitively assessed (Pyapali & Turner, 1996). Moreover, there are also at least two studies showing that aged humans have larger dendritic trees in the dentate and parahippocampal gyri compared with middle age adults, but again these were not cognitively characterized (Buell & Coleman, 1979; Flood *et al.*, 1985;).

Interestingly, in the mPFC, despite no overall age-related retraction, there was a similar, albeit with smaller magnitude, association between bigger apical dendritic trees in layer II/III Cg/PL pyramids and worse performance in the working memory (mPFC-dependent) test. Contrary to what happens for the HPC formation, until now, none of the analysis performed in the neurons of the mPFC had shown increased dendritic length in aged animals, when compared to young or middle age animals (for further detail see table 1 and 2 in chapter I).

As it was previously reported that neuronal dendritic remodelling is directly related with volumetric alterations (Cerqueira *et al.*, 2005 and 2007), we hypothesized that the above-mentioned findings should translate in equivalent volumetric changes in HPC and mPFC areas.

Volumetric alterations in these structures have already been coupled to the status of spatial learning (Rasmussen *et al.*, 1996; Rapp *et al.*, 1996 and 1999; Van Petten, 2004). The implicit link for aging is, of course, the existence of volumetric atrophy which impairs function. For instance, volumetric reductions in HPC and mPFC areas underpin age-related cognitive decline, in both humans (Jernigan *et al.*, 1991; Golomb *et al.*, 1993 Driscoll *et al.*, 2003 and 2009; Freeman *et al.*, 2008; Raz *et al.*, 2010) and rats (Rapp *et al.*, 1999; Driscoll *et al.*, 2006; Yates *et al.*, 2008). However, this view has been recently brought into question. For example, in humans, some studies reported no volumetric changes in the hippocampus during normal aging (Sullivan *et al.*, 1995; Raz, 1996) and another study suggested that larger hippocampi are associated with less effective memory performance in healthy young adults (Molnár &

Kéri, 2014). Of notice, these studies failed to accommodate the evidence of individual heterogeneity in aging. These contradictory findings could be justified by the fact that most of the studies performed in humans, relay on humans with pathology and that the heterogeneity observed in the aging brain has not been a focus of attention in previous volumetric studies.

Thereby, in the present work, after the cognitive characterization of both young and old animals as GPs and BPs, a detailed stereological analysis was applied to estimate the volumes of the main divisions of the HPC and mPFC.

First of all, it is important to mention that the present results document an enlargement of the HPC and mPFC volumes in the older rats when compared to the youngster counterparts. These data are against the majority of studies reported so far. Nevertheless, in accordance with our results, Ojo B. *et al.*, (2013) have shown that the volume of dorsal hippocampal CA3 increases with age. We cannot exclude, however, that alterations in astrocytic number, axonal myelination, and extracellular volume, could influence the observed differences between young and old individuals. Besides, our data was not normalized to the whole-brain volume as widely described in human studies (reviewed by van Petten, 2004).

Nevertheless, since our main goal was to understand the inter-individual differences between younger and older animals, and between GPs and BPs within each age category, age-related volume alterations will probably not interfere with our interpretations.

Herein, similar to the morphological data, we were able to correlate the behavioral differences, specifically the performance in the working and reference memory tasks, with volume measurements in the HPC and mPFC. Interestingly, our data revealed that, while reference memory performance (HPC-dependent task), in both groups, correlates with volumetric alterations in the HPC and mPFC, the working memory performance (mPFC-dependent task) was only related with alterations in the mPFC area.

As expected, and similar to what we just described in the morphometric analysis, our data also revealed that younger GPs had, compared with BPs, increased hippocampal volumes. In contrast, in aged animals this difference was reversed, with GPs presenting lower volume than BPs. In accordance with our findings regarding the older animals and in contrast to the common and intuitive belief that larger HPC are better, some studies surprisingly reported a negative correlation between cognitive abilities and HPC volume (Chantôme et al. 1999; Van Petten (2004), Molnár and Kéri 2014). Molnár and Kéri 2014, demonstrated that in individuals with Fragile X Syndrome, there is an inverse correlation between hippocampal volume and cognitive performance: larger HPC was associated with worse general memory, which is not present in age-matched individuals without pruning deficits. In addition, a meta-analysis from Van Petten (2004)

demonstrated also a negative correlation between hippocampal size and memory in young adults, whereas the correlation was positive in older participants.

Furthermore, these HPC volumetric alterations only occurred in the dorsal pole of the HPC axis. These results are in line with the different functions of the two hippocampi: the DHPC is involved in memory and cognitive processing, whereas the VHPC processes information related to the emotional and homeostatic states of the animal (Fanselow & Dong, 2010; McHugh *et al.*, 2011).

Furthermore, volumetric alterations in young animals were observed specifically in the DG granular layer and in the stratum radiatum of CA1 both in the dorsal pole of the hippocampal axis. A similar result was observed for aged animals, with alterations in the volume of the dorsal DG (molecular, granular and hilus layers) and dorsal CA1 (strata oriens, pyramidale and radiatum layers). Interestingly, no alterations in the CA3 area were observed between GPs and BPs, either in young or aged animals. Thus, it can be inferred that both the DG and CA1 were preferentially affected by age and could, therefore, be linked to alterations in spatial learning, while the CA3 region seems to be fairly resistant to the aging process. These particular data are, on one hand, in line with the research of Yang *et. al*, (2013) where they revealed that, unlike in CA1 synapses, the high frequency stimulation of the associative/commissural pathway leading to CA3 long-term potentiation is minimally affected by age; on the other hand, it is against the majority of studies that documented a role for CA3 in the acquisition of spatial memory in the Morris water maze task (Steffenach *et al.*, 2002; Jo *et al.*, 2007). These data then suggests that age-related changes may not be uniform across the hippocampus.

Our results showed that the behavioral correlates of mPFC volume were different for younger and older animals. Specifically, in younger animals, in general, the regional volume of mPFC positively correlates with working memory performance, while in older rats volumetric alterations negatively correlate with reference memory performance. Similar to the data described before, the direction of changes was similarly reversed between young and aged individuals, with young GPs presenting larger and old GPs smaller mPFC volumes. The areas responsible for the observed differences between the younger GPs and BPs animals were the PL and IL areas, which is in accordance with the role of these areas in working memory performance (Gisquet-Verrrier & Delatour, 2006). By contrast, in older animals volumetric alterations were essentially described in the Cg area. This area has been associated with remote memory and in conformity with this, volumetric changes of the Cg area correlate with reference memory performance (long-term memory task) (Teixeira *et al.*, 2006). Given this, and similarly to what happens in the hippocampus, the age-related volumetric changes may be different across the mPFC.

Taken together, it seems that during the normal aging process there is a relationship between dendritic extension and the volume of the HPC and mPFC. In young animals, better cognitive performance was associated with longer dendritic trees and increased HPC and mPFC volumes. This association was exactly the opposite in the aged group, in which better performance was associated with shorter dendritic branches and reduced volume on both brain structures.

In summary, during a normal aging process, our findings show, at the structural level, that important changes occur in the rat HPC and mPFC, and that these changes are related selectively to different cognitive performances, dependent on HPC or mPFC memory tasks. We propose that these findings, at least partly, explain the heterogeneity observed in the performance of both young and aged animals. Although there is still much that remains unknown, the theory that "bigger is always better" remains true for young animals, however, and apparently counterintuitive, for aged animals "smaller is better".

4.   The role of neurotrophins and autophagic activity in the regulation of dendritic growth/prunning

The association between larger dendritic trees and poorer cognitive function in older animals might be considered counter-intuitive. However, while bigger dendritic trees might mean more connectivity and better neuronal function, it is also well described that the accumulation of "waste" dendritic material and large dendritic trees, for example as a result of impaired autophagy and dendritic pruning deficits, hampers neuronal function and correlates with cognitive deficits, in both humans and animals (Tang *et al.,* 2014).

Dendritic pruning is a mechanism often used to selectively remove unnecessary and exuberant neuronal branches, not only in the immature nervous system (Tang *et al.* 2014) but also in the adult HPC (Gonçalves *et al.* 2016), thus ensuring the proper formation of functional optimized circuitries. Dendritic and synaptic pruning is highly dependent on autophagy-dependent protein turnover, as animals presenting constitutional (Tang *et al.* 2014) or induced (Crino, 2016) inhibition of autophagy have larger dendritic trees and increased spine density, which correlates with cognitive deficits. For example, in a study with adult diabetic animals, inhibition of autophagy was shown to exacerbate cognitive and synaptic plasticity deficits (Zhigui *et al.,* 2016). Also, in Fragile-X-syndrome patients, who have impaired dendritic pruning, there is also an inverse correlation between hippocampal volume and cognitive performance, which is not present in age-matched individuals without pruning deficits (Molnár & Kéri, 2014). According to some authors, this paradoxical negative correlation might be related to incomplete synaptic pruning

during childhood and adolescence, which refers to the elimination of unnecessary neurons and synapses to achieve more economic information processing (Foster *et al.*, 1999; Pohlack *et al.*, 2014); while other authors, in a more rudimentary way, explained this by the amount of effort provided by the subject: those who had difficulties performing the task tended to make greater effort and thus activated the associated structure(s) more, while those for whom the task was less difficult used more efficient strategies and needed less effort (Parks *et al.*, 1988).

In order to further dissect the mechanism that could contribute to the observed morphological and volumetric changes, and consequently to the inter-individual heterogeneity in cognitive aging, we analyzed the levels of autophagic activity in the HPC and mPFC. Furthermore, this was complemented with a quantification of the neurotrophin BDNF, and with pre- and post-synaptic markers (SYP, SNAP25 and PSD95). Our assessments showed that older cognitively impaired animals had reduced autophagic activity in both the dorsal HPC and the mPFC, when compared with older cognitively intact rats. Significantly, these observations were specific for older animals, as levels of autophagic markers in both brain regions did not differ between cognitively intact and cognitively impaired younger adult individuals. In most organisms, pathological aging is associated with decreased autophagic activity and autophagy inhibition induces degenerative changes that resemble those associated with aging (Rubinsztein, 2011). While the mechanisms of such relationship are far from being well understood, the most prevalent hypothesis considers a failure to clean toxic/waste protein debris, that accumulate with time and induce cellular dysfunction (Rubinsztein, 2011). In line with this, in the older cognitively impaired animals, decreased levels of autophagy are strongly and inversely correlated with the abundance of synaptic markers, a surrogate marker of dendritic length. Our findings, however, further extend the interpretation of the previous observations, by suggesting that in neurons, decreased autophagy results in less dendritic pruning and an accumulation of dendrites that hamper neuronal function. Moreover, this does not seem to be an inevitable consequence of aging, as levels of autophagic and synaptic markers in older cognitively intact individuals were strikingly similar to those of younger animals. Of note, BDNF levels, one of the main neurotrophins, similarly did not vary significantly with aging but were also increased in older cognitively impaired individuals, suggesting that increased dendritic growth, as well as decreased autophagic activity, might contribute to the increased dendritic length observed in these animals. Importantly, these correlations between autophagic activity, neurotrophin levels, synaptic markers and cognitive performance were region and task specific, as they were only present for reference memory (HPC-dependent) performance/HPC autophagic levels and working memory (mPFC-dependent)

156

performance/mPFC autophagic levels, respectively, further increasing the specificity and, therefore, the potential relevance of our findings.

Many factors could induce a decreased autophagic activity, similar to that presented by older cognitively impaired individuals. One of the best candidates is an enhanced activity of the mechanistic target of rapamycin (mTOR, formerly known as mammalian target of rapamycin complex), a redox/energy/nutrient sensor that inhibits autophagy and stimulates protein synthesis (Hands *et al.*, 2009). The activation of mTOR inhibits autophagy at an early step in autophagosome formation (Kim *et al.,* 2011). Increased mTOR activity (resulting in decreased autophagic activity) has been linked to cognitive dysfunction and learning deficits in a variety of disorders (Ehninger *et al.* 2008; Costa-Mattioli & Monteggia 2013), which are also associated with an increase in dendritic spines (Ehninger *et al.* 2009). More importantly, and in line with our results, lifelong treatment of mice (Majumber *et al.* 2012; Halloran *et al.* 2012) or accelerated senescence rats (Kolosova *et al.*, 2013) with the mTOR inhibitor rapamycin (that is considered an autophagy inducer) improved age-related cognitive dysfunction. Further adding to the work presented here, it would be valuable in the future to evaluate the levels of phospho-mTOR (p-mTOR), total mTOR (t-mTOR), phospho-S6 and total S6 in order to assess the role of mTOR-regulated autophagy as a determinant of age-dependent dendritic morphology.

Additionally, recent findings have also highlighted the role of microglia in synaptic pruning (Kim *et al.*, 2017). Microglia is a representative immune cell in the brain that constantly surveys the neural environment for pathogens, foreign materials and apoptotic cells. It is also responsible for the regulation of synaptic activity and maintenance of brain homeostasis (Weinhard *et al.*, 2018). Kim *et al.*, 2017 reported that deficient autophagy in microglia impairs synaptic pruning and causes deficits in neuropsychological behaviors.

Microglia is also affected by the aging process, which leads to various irregularities in their physiological functions, such as augmenting synaptic losses and altering brain plasticity, thereby affecting learning and memory and representing a strong risk for the development of neurodegenerative diseases (Weinhard *et al.*,2018).

Taken together, our results suggest that the inverse correlation between large dendritic trees and poor cognitive performance in the elderly, might at least be partly attributed to age-associated dendritic pruning deficits leading to larger and less efficient dendritic trees, possibly resulting in an increased volume. Of note, in the present work, the inverse correlations between dendritic tree length or volumetric alterations and cognitive performance are not present in the group of younger adults (in which an opposite trend is observed), supporting an age-related phenomenon. Nevertheless, as already discussed, a full

understanding of all these mechanisms can only be ascertained by added experimental manipulations of autophagic pathways.

In conclusion, our findings provide evidence that, in older animals, behavioral heterogeneity, dendritic length and volumetric differences can be ascribed to variations in neurotrophin levels and, more importantly, autophagic activity. To summarize these associations, we propose a model where the balance between neurotrophins and autophagic activity regulates dendritic growth/pruning thus contributing to the heterogeneity in the cognitive function of younger vs older animals (Figure 4). This data represents a paradigm shift in understanding the individual differences observed with aging.
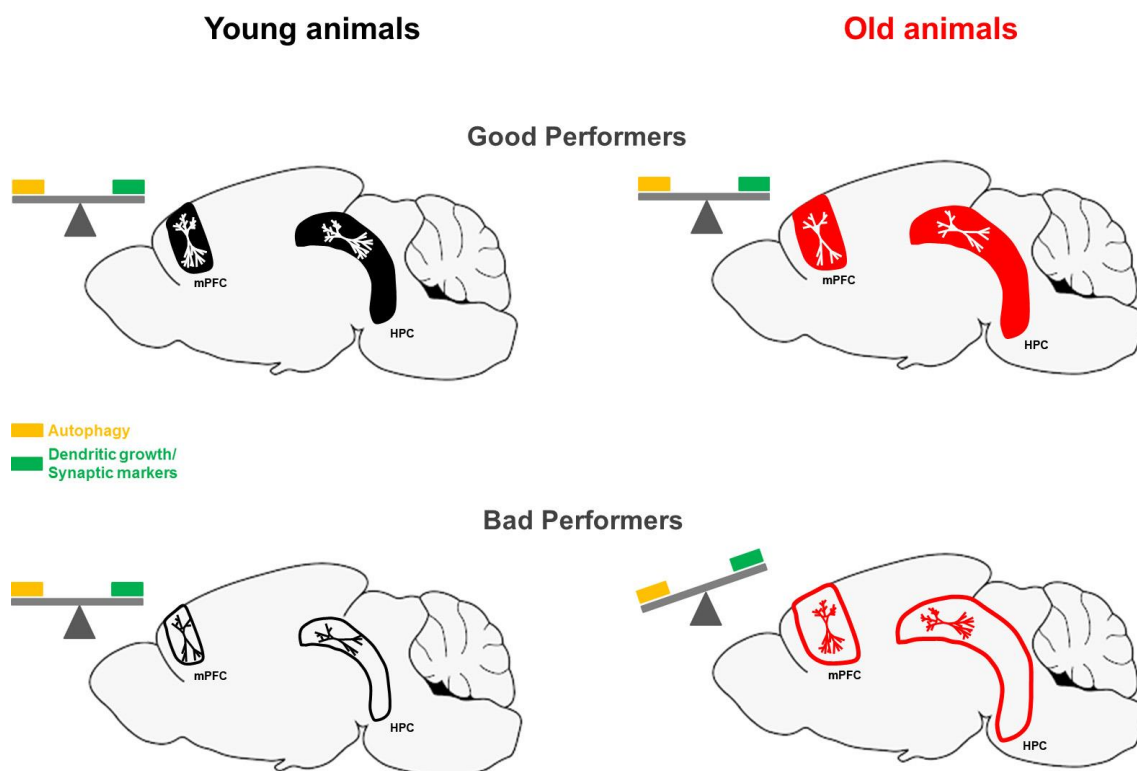
Figure 4| Schematic representation of the relations between age, cognitive performance, neuronal morphology, volumetric changes, autophagy, synaptic and dendritic growth markers, in the HPC and mPFC. In younger animals "bigger is better"; GPs have the biggest volumes and dendritic trees. However, there are no extensive differences in autophagy levels (LC3-II, p62), dendritic growth (BDNF) and synaptic markers (PSD95, SNAP25, SYP). In older animals, it seems that "smaller is better". BPs have the bigger volumes and dendritic trees, associated with a decrease in the levels of autophagy (LC3-II, p62), and an increase in dendritic growth (BDNF) and synaptic markers (PSD95, SNAP25, SYP).

5. Modulators of cognitive function: the role of cognitive training

Nowadays, cognitive training has been one of the strategies used to achieve successful cognitive aging (Willis *et al.*, 2006; Nouchi *et al.*, 2012; Jiang *et al.*, 2016).

Since the HPC is particularly vulnerable to aging (Mora *et al.*, 2007), in this study we used the HB as a cognitive training task (1-week protocol) (Van der Staay, 1999; Depoortère *et al.*, 2010).

Our work revealed that exposure to cognitive training in adulthood (10 months of age) prevented reference memory impairments later on. By contrast, no changes in working memory and behavioral flexibility skills were observed. These observations strengthen the view that training on specific "cognitive tasks" may serve to reinforce or reactivate circuits and therefore, to (at least partially) restore some of the age-induced deficits in cognition. In agreement with this data, Harburger *et al.*, (2007) reported that all enrichment treatments, including cognitive stimulation, improved spatial memory in aged females, indicating that either exercise or cognitive stimulation can improve memory in aged subjects. Interestingly, our work showed that the effects of a short period of cognitive training (1-week protocol) are long lasting in time. This points out that engagement in an highly cognitive demanding task, even for a relatively short period of time, is able to enhance cognitive function. In accordance, Arai *et al.*, (2009) demonstrated that when 15-day old mice were subjected to 2 weeks of EE, they exhibited enhanced hippocampal LTP. Later, when the same mice were breed and LTP was analyzed in their offspring, now reared in standard cages, hippocampal LTP was enhanced when compared to offsprings from non-enriched parents. Similarly, Cheng *et al.*, (2012) showed that in old adults ranging from 65 to 75 years of age, the effects of interventions on cognition are maintained 1 year after the training has ended.

Taken together, these findings could be explained by the cognitive reserve hypothesis, which rests on the ability of the brain to compensate for pathological changes associated with aging, depending on the previous stage of intellectual capability (Whalley *et al.*, 2004). This means that people with greater cognitive reserve can tolerate more the neurodegenerative brain changes associated with dementia or other brain pathologies, such as Parkinson's disease, multiple sclerosis, or stroke (Stern, 2012). Likewise, in our work, it seems that aged rats with higher cognitive reserve, as a consequence of the exposure to the HB test, cope better with age-related deterioration than the aged counterparts with lower cognitive reserve. Therefore, an important goal of aging research should be the promotion and sustainment of elderly people cognitive reserves.

Furthermore, our findings highlight the importance of the age at which individuals are exposed to cognitive stimulation and the duration of training (Frick, 2010). The influence of age to the exposure to EE seems to be controversial. Most studies indicate that EE at young ages appears to have even more beneficial

effects which is most likely due to the fact that brain development is not complete and more-long lasting molecular and anatomical changes are induced (Bouet *et al.*, 2011; Freret *et al.*, 2012). However, other studies found positive effects when EE training was initiated in already aged and cognitively impaired rodents (Bennett *et al.*, 2006). In accordance with this data, we also reported that 10 months of age seems to be the best sensitive period for the effectiveness of cognitive training, however we did not test animals younger than this time point.

Our goal was also to address the underlying mechanisms of cognitive training. It is known that cognitive stimulation induces structural and functional alterations in the rodent brain (Kolb *et al.*, 2003; Bindu *et al.*, 2007; Artola *et al.*, 2006, Freret *et al.*, 2012). Accordingly, a large body of literature has shown that exposure to an enriched environment enhances dendritic branching in several rodent cortical regions (Greenough & Volkmar *et al.*, 1973; Green *et al.*; 1983; Kolb *et al.*, 2003; Bindu *et al.*, 2007). However, some studies failed to see such differences (Diamond *et al.*, 1976), while others reported differences in dendritic branching in dentate granule cells, but only in female EE animals (Juraska *et al.*, 1985, 1989). Regarding volumetric changes, an increase in cortical thickness was also seen after cognitive training (Mohammed *et al.*, 2002). Nevertheless, these reports failed to accommodate the inherent heterogeneity of cognitive abilities, as well as the characteristic heterogeneous process of cognitive aging (Mota *et al.*, 2018).

In the present work, structural analysis confirmed a change in neuronal length and HPC volume of worse HB performing animals that reached the level of the best performing group. Also, no significant effects of training were reported for the dendritic length or HPC volume. These findings are in contradiction with the majority of studies using EE animal models, which report that exposure to an enriched environment enhances dendritic branching (Greenough & Volkmar *et al.*, 1973; Green *et al.*, 1983; Kolb *et al.*, 2003; Bindu *et al.*, 2007). Of note, most of these structural studies were performed in adult rats, which made it difficult to compare with our own data. Nevertheless, our results are in accordance with our previous data showing that in aged individuals "smaller is better" (Mota *et al.*, 2018), as the cognitively trained BP group reached the level of the best performing animals. Accordingly, aged GP untrained individuals had smaller DG and CA1 dendritic trees and smaller HPC volume when compared to BP untrained animals. These similar outcomes, between successfully aged GPs and cognitively trained aged animals, further allow us to hypothesize that similar mechanisms might underpin both processes.

Neurons are long-lived cells with considerable specialized membrane and protein turnover, thereby rendered highly susceptible to neurotoxic insults resulting from inefficient debris removal. Not surprisingly, an inefficient autophagic process leads to neurodegeneration (Hara *et al.*, 2006; Komatsu *et al.*, 2006).

Of notice, EE was shown to enhance autophagy in the rat hippocampus (Takahashi *et al.*, 2014). These pivotal studies, together with our own work, shed light to an emerging concept of healthy aging, where neuronal maintenance, through successful autophagic debris removal, allows life-long optimal cognitive function.

As already discussed, care must be taken when one tries to parallel the outcomes of animal and human EE. Education, literacy, complex leisure behaviors and sustained physical activity are all factors influencing human cognitive reserve that seem, at first glance, difficult to model in animal paradigms. However, animal models of EE have been shown to strengthen the cognitive, social, and physical components as would occur in a socially and intellectually active human lifestyle (Richards *et al.*, 2005; Petrosini *et al.*, 2009). Accordingly, several human studies recapitulate findings in animal models of EE. Thus, as seen in rodents, EE enhances dendritic branching in humans (Jacobs *et al.*, 1993). Similarly, healthy elderly human cognitive training parallels the increase in cortical thickness found in rats (Jiang *et al.*, 2016; Seider *et al.*, 2016), while aerobic exercise was also shown to reverse age-related decreases in human hippocampal volume, which correlated with an improvement in spatial skills (Erickson *et al.*, 2011). Although none of these translational studies directly addressed the central question of cognitive heterogeneity and its underlying mechanisms, the similarity in the findings between existing human and animal studies, partially validates our animal model paradigm strengthening the need for further following studies both in humans and animals.

In conclusion, our work suggests that, at least in rodents, the HB test is a useful tool to enhance cognitive function, and its effects are circuitry-specific and long lasting in time. The beneficial effects of cognitive training are potentially mediated by alterations in dendritic branching. These promising findings highlight the existence of continuous functional plasticity, which brings optimism about the possibility of promoting "mindspan".

In this thesis we demonstrated the heterogeneity of cognitive aging in a large set of old and young male Wistar Han rats, while pinpointing structural and molecular correlates of this functional heterogeneity. Additionally, our findings suggest that cognitive training is an important modulator of cognitive function during the normal aging process.

In summary, the main achievements of the work developed in this thesis indicated that:

1) Aged rats, on average, showed poorer spatial learning and behavioral flexibility than young adults while, interestingly, the degree of cognitive decline was highly variable in both groups.

2) Behavioral heterogeneity, dendritic length and volumetric differences associated to aging can be ascribed to variations in neurotrophin levels and, more importantly, autophagic activity.

3) Cognitive training significantly prevented age-induced cognitive impairments.

4) Even a relatively short period of exposure to cognitive training is sufficient to improve cognitive performance, mainly within brain circuits involved in the specific training task.

Hopefully, these studies will contribute to understand the individual differences observed with aging, not only in rodents, but also in humans. We expect our work will help promoting the exploration of novel approaches to prevent or restore cognitive impairments, which might contribute for a successful aging and an increased "mindspan".

The conclusions withdrawn from this work showed that behavioral heterogeneity, dendritic length and volumetric differences associated to aging can be ascribed to variations in neurotrophin levels and, more importantly, to autophagic activity. It also showed that cognitive training is an important determinant of cognitive function. While these findings contribute to a better understanding of the aging process, at the same time, they also raise new questions that merit further investigation.

One of the main achievements of this work was to ascertain that alterations in the dendritic architecture of neurons from the HPC and mPFC, as well as volumetric changes of these areas, correlate with individual differences in cognitive abilities in both old and young animals. These structural alterations were only addressed in post-mortem tissues of specific brain areas at a single time point (aged animals: 22-24-month-old and young animals: 4-6-month-old). Thus, a global view of the rat brain across the lifespan could lead to new insights related to normal brain aging. A natural follow-up of this work would thus be a longitudinal study of old animals with different structural and cognitive evaluations at different time points. To achieve this goal, high-resolution brain imaging techniques including magnetic resonance imaging, multiphoton imaging and array tomography, could be used to examine the function, structure and volumes of brain areas in the aging brain.

One of the hypothesis proposed in this work is that alterations in dendritic architecture as a consequence of the aging process are related with autophagic activity and consequently with changes in dendritic pruning. However, a causal relation between autophagy and cognitive aging needs to be confirmed. To answer this question, experimental manipulations of autophagy levels (preferably in adulthood) and a subsequent cognitive characterization of the animals as they age is of interest.

Other functional correlates assessed by means of electrophysiological recordings would also be a valuable aid to the conclusions of the present thesis. Accordingly, we envision *ex vivo* and freely moving recordings are relevant as they will be able to show reliable links between functional neural activity and molecular, morphological or behavior readouts. Also, *in vivo* two-photon imaging, head-mounted fluorescent microscopes in freely moving animals, and optogenetics, could be used to better define the cellular neurosystems involved in memory.

Cognitive training in adulthood was able to improve/prevent age-related cognitive impairments. Furthermore when animals were divided into GPs and BPs, structural analysis confirmed a reversal in neuronal length and HPC volume of the worse performing animals that reached the level of the best performing group. In this setup, alterations in neurotrophin levels and autophagic markers should also be addressed in order to check their role in cognitive training-dependent modulation of cognitive function. In addition, questions concerning the most efficient period to perform the cognitive training task and how many times it's necessary to repeat the task should also be further dissected.

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173

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ANNEXES

SCIENTIFIC REP🅞RTS

Structural and molecular correlates of cognitive aging in the rat

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Aging is associated with cognitive decline. Herein, we studied a large cohort of old age and young adult male rats and confirmed that, as a group, old rats display poorer spatial learning and behavioral flexibility than younger adults. Surprisingly, when animals were clustered as good and bad performers, our data revealed that while in younger animals better cognitive performance was associated with longer dendritic trees and increased levels of synaptic markers in the hippocampus and prefrontal cortex, the opposite was found in the older group, in which better performance was associated with shorter dendrites and lower levels of synaptic markers. Additionally, in old, but not young individuals, worse performance correlated with increased levels of BDNF and the autophagy substrate p62, but decreased levels of the autophagy complex protein LC3. In summary, while for younger individuals "bigger is better", "smaller is better" is a more appropriate aphorism for older subjects.

Aging is a process that, even in healthy individuals, is generally linked to a decline in cognitive abilities[1]. However, one of the most striking characteristics of human aging is its heterogeneity[2,3], with some individuals maintaining a preserved cognitive function until late in life. Since cognitive ability is a crucial determinant of elderly people's quality of life, a thorough understanding of the mechanisms underlying its heterogeneity is of paramount importance. To this purpose, we decided to study a large cohort of young adults (4–6 month-old) and older rats (22–24 month-old).

Rats have been intensively used as models of cognitive aging[4-7]. A large body of literature indicates that, as in humans, spatial learning and memory tasks in rodents also require the hippocampus (HPC) and the medial prefrontal cortex (mPFC), and typically display performance decrements across the lifespan[5,6,8]. In fact, older male rats, approximately 22–24 months-old, show impairments in several spatial memory tasks including: Y and T mazes[9], radial arm maze[10], Morris water maze[6-8], water radial arm maze[11] and Barnes maze[12].

The well-documented age-related behavioral deficits are concomitant, and seem to be correlated with morphological alterations in brain structure. It is widely accepted that aging is accompanied by an overall brain volume loss, in both humans[13-16] and rats[17,18], that accompanies the decline in cognitive function. Moreover, several studies reported age-related cognitive decline to be associated with volume loss and dendritic atrophy in areas implicated in cognitive abilities, such as the HPC and the mPFC[13,18-21].

The homeostasis of the mammalian neuroarchitecture is a dynamic process involving a balance between sprouting and pruning. The mechanisms underlying these processes are particularly active during development and pathological neurodegeneration[22,23] but are also functional in physiological conditions. Of note, while in equilibrium, the relative importance of each of these processes varies throughout the lifespan, with synapse and dendritic formation generally exceeding pruning during brain development, and an opposite trend occurring in the adult brain[23,24]. Importantly, the maintenance of this balance is under tight control through protein synthesis and autophagic recycling[25-26].

Herein, we explored cognitive aging and its structural and molecular correlates in a large set of old and young male Wistar Han rats. The results show that while for younger individuals "bigger is better", it seems that "smaller is better" is more appropriate for older subjects, as older animals with smaller dendritic trees, increased neuronal autophagy and decreased brain-derived neurotrophic factor (BDNF) and synaptic markers, presented the best performances.

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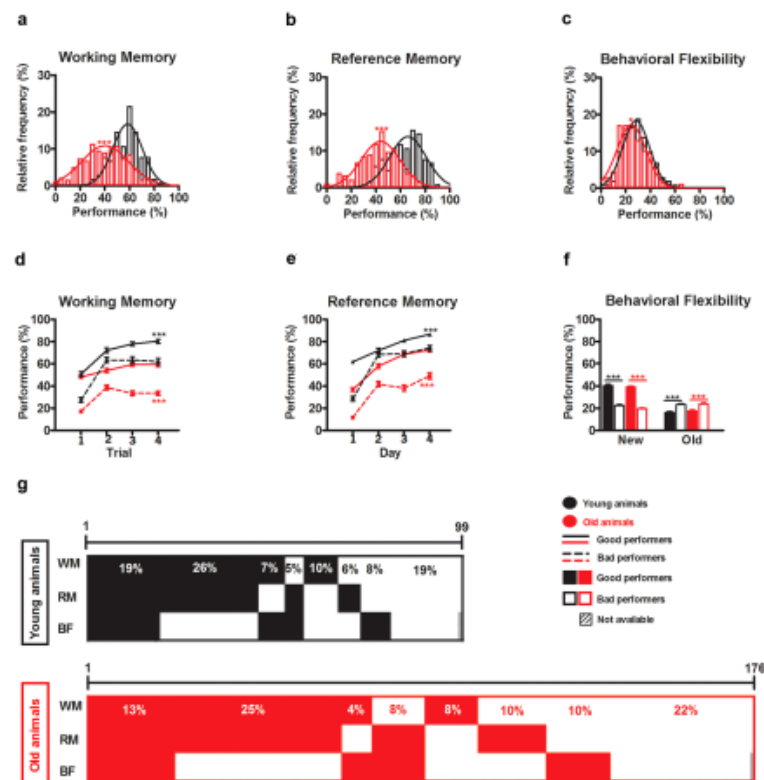179

Behavioral assessment

Figure 1. Behavioral assessment and performance clustering of younger and older rats. When all animals are considered: (**a**) The working memory (older n = 176; younger n = 102) and (**b**) reference memory (older n = 176; younger n = 102) PIs of older rats are worser and broader than that of youngsters. (**c**) The performance in behavioral flexibility (older n = 176; younger n = 101) is less variable but maintains the previous trend as older rats are the worst performing group. When similar age animals are clustered (see methods for details) in GPs and BPs: GP and BP clusters had significantly different learning curves in (**d**) working memory (older: GPs n = 89 BPs n = 87; younger: GPs n = 63 BPs n = 39) and (**e**) reference memory tasks (older: GPs n = 99 BPs n = 77; younger: GPs n = 61 BPs n = 41). (**f**) GPs spent more time in the new and less in the old quadrants of the behavioral flexibility task (older: GPs n = 62 BPs n = 114; younger: GPs n = 40 BPs n = 61). Interestingly, (**g**) the frequencies of the different patterns were similar in younger and older animals. Continuous lines in (**a–c**) are Gaussian fits. Error bars represent SEM; *p < 0.05; ***p < 0.001.

Results

Age is associated with cognitive decline and behavioral heterogeneity.   Older animals displayed a worse cognitive performance in all tested domains (working memory: $t(267) = -10.122$, $p < 0.0005$, $d = 1.148$; reference memory: $t(276) = -11.274$, $p < 0.0005$, $d = 1.403$; behavioral flexibility: $t(275) = -2.222$, $p = 0.027$, $d = 0.277$) (Fig. 1a–c). Moreover, the performance of older rats in working and reference memory was more heterogeneous than that of younger rats (working memory variance: older = 290.33%^{2} vs. younger = 141.12%^{2}; reference memory variance: older = 262.47%^{2} vs. younger = 180.47%^{2}), while no differences were observed in the distributions of behavioral flexibility performances of older and younger animals (variance: older = 128.96%^{2} vs. younger = 118.56%^{2}) (Fig. 1a–c).

180

A k-means clustering was performed to classify younger and older animals according to their performance in working memory, reference memory or behavioral flexibility tasks. This resulted, for each test and age, in two groups of subjects (good performers – GPs and bad performers – BPs), which had significantly different performances (Table 1, Fig. 1d–f; see also the Supplementary Fig. S1 for the escape latency to find the platform (s)). A two-way ANOVA revealed a significant effect of age and performance, as a significant interaction between these two factors, both in the working and reference memory tests (Table 1, Fig. 1d–f). Overall, in memory tests, younger adult rats performed better than older rats, with younger GPs being the best (Tukey's test $p < 0.001$), younger BPs and older GPs intermediate, and older BPs the worst performers (Fig. 1d,e). In contrast, although the performance in the behavior flexibility task was significantly different between age categories (only for the time spent in the new quadrant) and performance groups, performance of younger and older GPs was similar, as was that of younger and older BPs (Tukey's test $p > 0.05$), without a significant interaction between age and performance group (Table 1, Fig. 1f).

A subsequent analysis of each individual's cluster membership for each of the behavioral tests revealed that these were poorly correlated, as animals were distributed across all possible combinations of GPs and BPs, without a clear predominance of impaired cognitive performance across all tests. Importantly, although cognitive performance patterns were widely variable between individuals of the same age group, for each age group, the same eight different patterns of performance were identified, and their overall distribution was approximately similar across the different ages (Fig. 1g). Of notice, the percentage of animals belonging to the GPs group in all the tests was lower in older animals when compared with younger animals (older = 13%, younger = 19%), while the proportion of animals belonging to BPs in all the tests was similar between the different age groups (older = 22%, younger = 19%).

Age triggers dendritic atrophy that correlates with individual cognitive performance.
To better understand the above described behavioral differences, we analyzed the morphology of dorsal HPC neurons (dentate gyrus (DG) granular, cornus ammonis (CA) 3 and CA1 pyramidal neurons). Considering the granular neurons of the HPC, older animals presented shorter dendritic trees when compared to younger animals ($t(37) = -8.314$, $p < 0.0005$, $d = 2.736$, Fig. 2a). Regarding CA3 and CA1 HPC pyramidal neurons, apical dendrites presented an age-dependent decrease in dendritic length whereas basal dendrites presented no differences in CA1 neurons but an increased dendritic length in CA3 pyramidal neurons (CA3 apical dendrite $t(37) = -2.907$, $p = 0.006$, $d = 0.807$; CA3 basal dendrite $t(28) = 3.968$, $p = 0.0004$, $d = 1.027$; CA1 apical dendrite $t(38) = -7.282$, $p < 0.0005$, $d = 1.914$; CA1 basal dendrite $t(40) = -1.714$, $p = 0.094$, $d = 0.552$; Fig. 2b,c).

Morphological analysis of HPC neurons, in both young and older animals, revealed a correlation between dendritic length and the individual performances in the reference memory task. Curiously, while for younger animals the apical dendritic trees of DG and CA1 neurons presented a significant positive correlation (DG: $r^2 = 0.714$, $p = 0.003$; CA1: $r^2 = 0.523$, $p = 0.046$), in older animals this correlation was reversed, with better performers having the smallest apical trees in all three regions analyzed (DG: $r^2 = -0.659$, $p < 0.0005$; CA3: $r^2 = -0.450$, $p = 0.027$; CA1: $r^2 = -0.572$, $p = 0.002$) (Fig. 2d,h,l). We found a similar negative correlation, in older, but not younger animals, between working memory performance and dendritic tree length of DG, CA3 (apical) and CA1 (apical and basal) cells (Supplementary Table S2). Significantly, there was no correlation in older animals between behavioral flexibility performance (a more mPFC-dependent task) and dendritic length of any HPC cell type (Supplementary Table S2; for additional information regarding individual animal performance in all cognitive tasks see Supplementary Fig. S3a–c).

To further clarify the relationship between HPC dendritic length, age and reference memory performance, we conducted a two-way ANOVA using the clustering of animals according to performance on this test. This analysis revealed a significant effect of age, but not of performance group, and a significant interaction between the two factors, in the average dendritic length of granular neurons and the apical tree of CA1 pyramidal cells (Table 1, Fig. 2e,m). In contrast, no factor seemed to influence the length of CA1 pyramidal cell basal dendrites (Table 1). Regarding comparisons within age groups, old BPs presented a significant increase in the dendritic length of both granular and apical dendrite of CA1 pyramidal neurons when compared with old GPs (DG: $t(22) = -2.632$, $p = 0.015$, $d = 1.033$; CA1 apical dendrite: $t(25) = -3.312$, $p = 0.003$, $d = 1.382$) (Fig. 2e,m). Also, regarding granular neurons, a significant difference was observed between young GPs and BPs. Here, GPs display higher dendritic lengths when compared with BPs ($t(13) = 3.540$, $p = 0.004$, $d = 1.952$) (Fig. 2e). Data on CA3 pyramidal neurons revealed a significant effect of age, but not of performance group nor any interaction in the length of both basal and apical dendrites (Table 1, Fig. 2i).

To explore in which parts of the dendritic tree laid the above-mentioned differences, we performed a Sholl analysis, which measures the number of intersections as a function of distance from the soma. Results for granular dendrites revealed a significant effect of age, but not of performance group, and a significant interaction between the two (Table 1, Fig. 2f). Repeated measures ANOVA revealed that younger GPs, when compared with the BP group, had an overall increase in the number of intersections ($F_{(1,13)} = 11.050$, $p = 0.005$, $\eta p^2 = 0.459$), both proximally and distally (Fig. 2f). Results of the Two-way ANOVA analysis for CA3 and CA1 apical dendrites revealed no significant effect of age, performance, neither an interaction between these two factors (Fig. 2j,n). However, group comparisons revealed an overall increase in the number of intersections in apical CA1 dendrites of young GPs when compared with the BP group ($F_{(1,13)} = 6.294$, $p = 0.026$, $\eta p^2 = 0.326$) (Fig. 2n). These alterations observed in HPC neurons are exemplified in the reconstructions of Fig. 2g,k,o.

Since some of the cognitive tasks assessed in this work were mPFC-dependent, we also analyzed the morphology of mPFC neurons (cingulate/prelimbic (Cg/PL) and infralimbic (IL) pyramidal neurons; Supplementary Fig. S4). We found an age-dependent reduction in the length of basal dendrites of Cg/PL pyramidal neurons, but no major changes in other dendritic domains (Supplementary Fig. S4a,b). Pearson correlations between behavior performance and dendritic length showed a significant association between working memory and the apical

181

	Behavioral assessment (Fig. 1d–f)										
	Working Memory (number of GPs, BPs)										
		df	*F*	*P*	*ηρ²*						
Repeated measures	Older animals (GPs n = 89, BPs n = 87)	1,174	236.373	<0.0005	0.576						
	Younger animals (GPs n = 63, BPs n = 39)	1,100	97.730	<0.0005	0.494						
	Reference Memory (number of GPs, BPs)										
	Older animals (GPs n = 99, BPs n = 77)	1,174	181.672	<0.0005	0.511						
	Younger animals (GPs n = 61, BPs n = 41)	1,100	79.790	<0.0005	0.444						
	Behavioral Flexibility - New Quadrant (number of GPs, BPs)										
		df	*t*	*P*	*d*						
t-test	Older animals (GPs n = 62, BPs n = 114)	174		<0.0005	2.882						
	Younger animals (GPs n = 40, BPs n = 61)	99	13.685	<0.0005	2.738						
	Behavioral Flexibility - Old Quadrant (number of GP, BP)										
	Older animals (GPs n = 62, BPs n = 114)	158	5.047	<0.0005	0.761						
	Younger animals (GPs n = 40, BPs n = 61)	99	−5.217	<0.0005	1.063						

	Behavioral assessment (Fig. 1d–f)		Performance			Age			Interaction		
		df	*F*	*P*	*ηρ²*	*F*	*P*	*ηρ²*	*F*	*P*	*ηρ²*
	Working Memory	1,274	273.651	<0.0005	0.500	245.073	<0.0005	0.472	10.753	0.001	0.038
	Reference Memory	1,274	213.618	<0.0005	0.438	238.943	<0.0005	0.466	10.141	0.002	0.036
	Behavioral Flexibility										
	New quadrant	1,273	499.625	<0.0005	0.647	6.291	0.013	0.023	0.823	0.365	0.003
	Old quadrant	1,273	42.565	<0.0005	0.135	0.738	0.391	0.003	0.153	0.696	0.001
	Morphological analysis - Hippocampus (Fig. 2e,i,m)										
Two-way ANOVA	Granular Neurons	1,35	3.276	0.079	0.086	67.480	<0.0005	0.658	22.030	<0.0005	0.386
	CA3 pyramidal neurons (apical tree)	1,36	2.513	0.112	0.065	4.784	0.035	0.117	0.183	0.671	0.005
	CA3 pyramidal neurons (basal tree)	1,36	2.586	0.116	0.067	11.326	0.002	0.239	0.763	0.388	0.021
	CA1 pyramidal neurons (apical tree)	1,38	2.242	0.143	0.056	29.918	<0.0005	0.441	9.781	0.003	0.205
	CA1 pyramidal neurons (basal tree)	1,38	3.356	0.075	0.081	2.045	0.161	0.051	0.554	0.461	0.014
	Sholl analysis - Hippocampus (Fig. 2f,j,n)										
	Granular Neurons	1,35	3.482	0.070	0.090	44.316	<0.0005	0.559	13.869	0.001	0.284
	CA3 pyramidal neurons (apical tree)	1,36	3.022	0.091	0.077	2.254	0.142	0.059	1.049	0.313	0.028
	CA1 pyramidal neurons (apical tree)	1,38	0.059	0.809	0.002	3.546	0.067	0.085	2.456	0.125	0.061
	Western Blot Data - Hippocampus (Fig. 3a,b,d–g)										
	LC3-II	1,30	9.964	0.004	0.270	1.112	0.299	0.040	0.270	0.608	0.010
	P62	1,30	1.101	0.303	0.039	1.545	0.225	0.054	1.930	0.176	0.067
	BDNF	1,30	0.526	0.475	0.019	0.128	0.724	0.005	2.126	0.156	0.073
	PSD95	1,30	1.876	0.182	0.065	2.066	0.162	0.071	1.175	0.288	0.042
	Synaptophysin	1,29	1.626	0.214	0.059	0.012	0.913	0.000	2.558	0.122	0.090
	SNAP25	1,30	0.595	0.447	0.022	2.202	0.149	0.075	4.944	0.035	0.155

Table 1. Results of repeated measures, *t*-test and two-way ANOVA on the data obtained from younger and older animals.

dendritic length of Cg/PL, that was positive in younger subjects ($r^2 = 0.630$, p = 0.012) and negative in older animals ($r^2 = -0.504$, p = 0.007) (Supplementary Fig. S4c). The performance in the reference memory task was only significantly negatively correlated with the apical dendritic length of IL pyramidal neurons of older animals ($r^2 = -0.465$, p = 0.029) (Supplementary Fig. S4o). Regarding the behavioral flexibility task, only in younger animals a positive correlation was found between this task and the apical dendritic length of IL pyramidal neurons ($r = 0.611$, p = 0.046; Supplementary Table S5; for additional information regarding individual animal performance in all cognitive tasks see Supplementary Fig. S3e,f). In addition to individual correlations, we performed within group comparisons (older and younger GPs and BPs for each task) on average dendritic lengths and distribution of dendritic processes. Interestingly, differences were only present between IL neurons of GPs and BPs in younger animals ($t(10) = 2.377$, p = 0.039, $d = 1.550$) (Supplementary Fig. S4p). Finally, the distribution of dendritic processes resulting from Sholl analysis in mPFC neurons showed a significantly more ramified apical

182

Morphological analysis - Dorsal HPC neurons

Young vs old animals

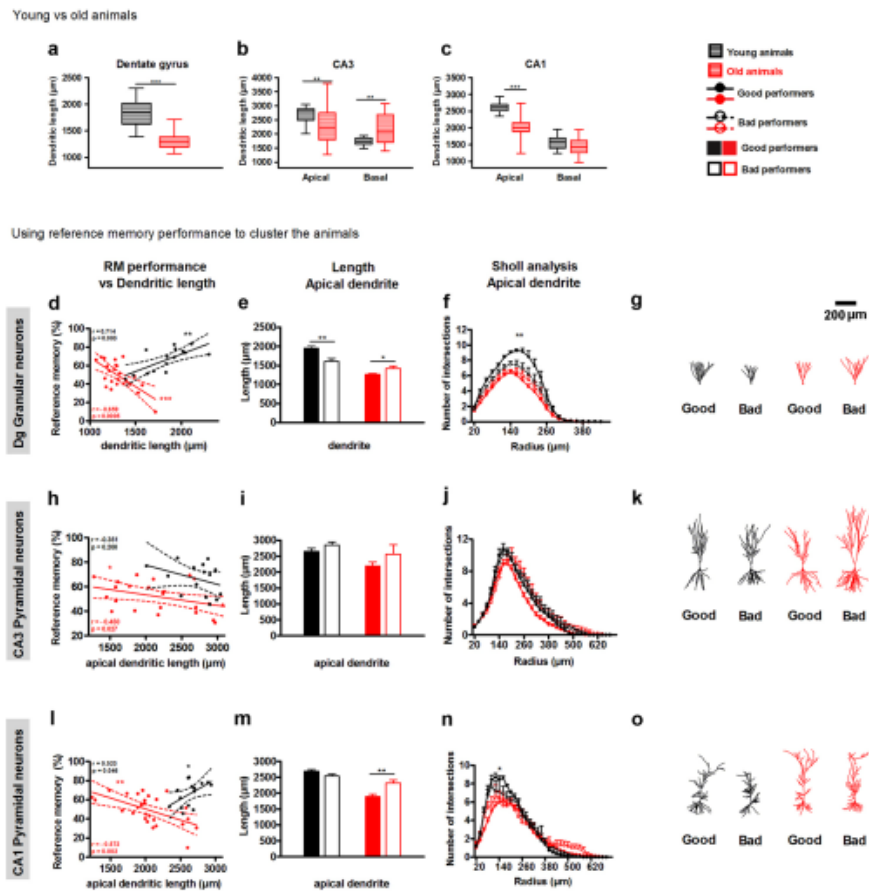Using reference memory performance to cluster the animals

Figure 2. Morphological analysis of HPC neuron dendritic arborizations. When a random sample of all animals is considered (older = 27; younger = 15). (a–c) Comparison of dendritic lengths of DG granular, CA3 and CA1 pyramidal neurons between younger and older rats. (d) Correlation between granular neuron dendritic lengths and individual performances in the reference memory task of both younger and older rats. When similar age animals are clustered (see methods for details) in GPs and BPs according to reference memory performance (older GPs = 16 (18 for CA1); older BPs = 8 (9 for CA1); younger GPs = 10; younger BPs = 5). (e) Average dendritic lengths for both GPs and BPs of younger and older animals. (f) Sholl analysis of the apical dendrite of DG granular neurons. This graph presents the mean number of intersections of apical dendritic branches with consecutive 20 μm spaced concentric spheres. (g) Representative reconstructions of DG granular neurons used in the previous analysis. (h–k) The same analysis was performed for CA3 pyramidal neurons and in (l–o) for CA1 pyramidal neurons. Error bars represent SEM, doted lines represent confidence intervals and continuous lines are linear fits; $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. (RM – reference memory).

dendritic tree of IL pyramidal neurons in younger GPs as compared with younger BPs ($F_{(1,10)} = 9.339$, $p = 0.012$, $\eta\rho^2 = 0.483$), with no differences in the other parameters (Supplementary Fig. S4q). For a comprehensive overview of the simultaneous alterations occurring at different brain regions, Supplementary Fig. S6 depicts the individual morphological alterations of the analyzed animals, including animal performance and respective relative dendritic lengths of DG, CA3, CA1, Cg-PL and IL brain regions.

183
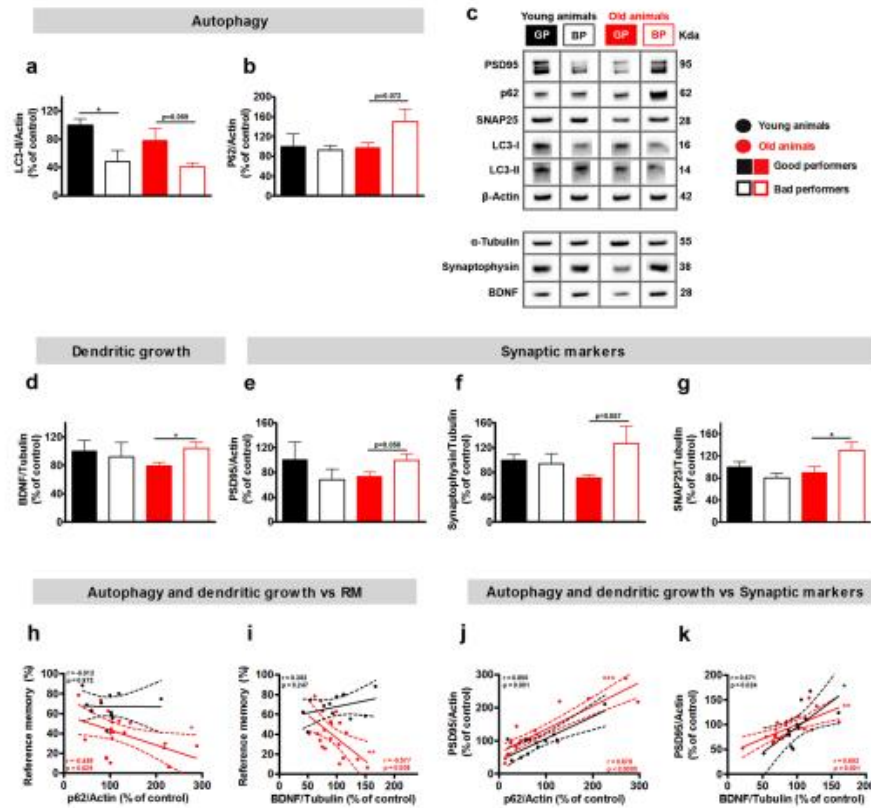
Western blot analysis - Dorsal HPC

Figure 3. Defective autophagy signaling and dendritic pruning in the HPC of older BPs. Performance in reference memory was used to cluster (see methods for details) both younger and older animals as GPs and BPs. A random sample of these were used for molecular analyses (younger GPs = 5–6; younger BPs = 5; older GPs = 10; older BPs = 9–10). (a,b) Levels of autophagy markers, LC3-II (a) and p62 (b), normalized to actin. (d) BDNF levels normalized to tubulin. (e–g) Levels of synaptic markers PSD95, SYP, and SNAP25 normalized to actin, tubulin, and tubulin, respectively. (c) Representative western blots of PSD95, p62, SNAP25, LC3, Actin, Tubulin, SYP, and BDNF. For each protein, the blots were cropped from different parts of the same gel. (h,i) Correlation between RM performance and p62 or BDNF levels, respectively. (j,k) Correlation between PSD95 and p62 or BDNF levels, suggesting a relationship between the levels of synaptic markers and autophagy or dendritic growth, respectively. Error bars represent SEM, doted lines represent confidence intervals and continuous lines are linear fits; *p < 0.05; **p < 0.01; ***p < 0.001. (RM – reference memory).

Impaired autophagy impacts on dendritic structure. Autophagic activity has been identified as a critical mechanism underlying dendritic remodeling[25]. To test the hypothesis that autophagy signaling is disrupted in aging and could be associated with alterations in dendritic recycling in the HPC, we performed western blot analysis of the autophagosome markers: microtubule-associated protein 1A/1B-light chain 3 (LC3) and nucleoporin 62 (p62). To determine the relationship between autophagic activity and dendritic size, protein levels of BDNF were analyzed. As for structural markers of synaptic function, the levels of postsynaptic density protein 95 (PSD95), synaptosomal-associated protein 25 (SNAP25) and synaptophysin (SYP) were determined (Fig. 3c).

184

To test the association between dendritic length and autophagy/neurotrophin levels, the levels of the synaptic marker PSD95 (a surrogate marker of dendritic extension) was correlated with both p62 (whose increased levels represent decreased autophagic activity) and BDNF. In both younger and older animals, HPC levels of PSD95 were positively correlated with p62 (younger: $r = 0.850$, $p = 0.001$; older $r = 0.878$, $p < 0.0005$; Fig. 3j) and BDNF (younger: $r = 0.671$, $p = 0.024$; older $r = 0.692$, $p = 0.001$; Fig. 3k).

Two-way ANOVA revealed a significant effect of reference memory performance, but not of age, neither an interaction between them, in the HPC levels of LC3-II, but not of p62 (Fig. 3a,b; Table 1). Group comparisons further revealed that, in younger animals, the levels of LC3-II in the HPC were significantly lower in BPs than in GPs ($t(9) = 2.988$, $p = 0.015$, $d = 1.750$; Fig. 3a), while the levels of p62 in the HPC were similar between the two groups. In older animals, there was a trend toward BP animals having less HPC LC3-II ($t(18) = 2.020$, $p = 0.059$, $d = 0.903$); Fig. 3a) and more p62 ($t(18) = -1.912$, $p = 0.072$, $d = 0.855$; Fig. 3b). At the individual level, younger animals had a significant positive correlation between performance in the reference memory task and the level of HPC LC3-II ($r = 0.790$, $p = 0.004$), but not of p62, while in older rats, there was a significant correlation between reference memory performance and the levels of both HPC LC3-II ($r = 0.523$, $p = 0.018$) and p62 ($r = -0.489$, $p = 0.029$; Fig. 3h) (see also Supplementary Table S7). There were no significant correlations between any HPC autophagy markers and performance in the working memory or behavioral flexibility tasks (Supplementary Table S7).

Hippocampal BDNF protein levels were similar in younger BPs and GPs, but were significantly higher in older BPs than in older GPs ($t(18) = -2.500$, $p = 0.022$, $d = 1.118$; Fig. 3d). In younger animals, there was also a trend toward a positive correlation between HPC BDNF levels and performance in working memory ($r = 0.610$, $p = 0.061$) but not in the reference memory task ($r = 0.382$, $p = 0.247$; Fig. 3i) nor in the behavioral flexibility task (Supplementary Table S7). In older rats, HPC BDNF levels were significantly negatively correlated with the performance in reference and working memory tasks (reference memory: $r = -0.577$, $p = 0.008$; Fig. 3i; working memory: $r = -0.447$, $p = 0.048$) but not the behavioral flexibility task (Supplementary Table S7).

Regarding synaptic markers, only HPC SNAP25 levels (Fig. 3g, Table 1) had a significant interaction between age and performance group, without a significant effect of either factor alone. In addition, HPC levels of PSD95 (Fig. 3e), SYP (Fig. 3f) and SNAP25 (Fig. 3g) were different between GPs and BPs solely in older animals. In line with data from BDNF, a significant increase was observed in the levels of SNAP25 and a trend towards an increase of PSD95 and SYP in older BPs, when compared with older GPs (SNAP25 $t(18) = -2.192$, $p = 0.042$, $d = 0.980$; PSD95 $t(18) = -2.045$, $p = 0.056$, $d = 0.914$; SYP $t(17) = -2.042$, $p = 0.057$, $d = 0.912$). Lastly, concerning older animals, HPC PSD95 levels showed a significant negative correlation with reference ($r = -0.448$, $p = 0.048$) and working memory ($r = -0.513$, $p = 0.021$) performances, while HPC SNAP25 levels presented solely a significant negative correlation with the performance in the reference memory task (reference memory task: $r = -0.560$, $p = 0.010$, working memory task: $r = -0.339$, $p = 0.143$). For HPC SYP levels, a trend toward a negative correlation with reference memory ($r = -0.453$, $p = 0.052$) and working memory tests ($r = -0.406$, $p = 0.085$) was observed. No correlations were found between synaptic markers and the performance in the behavioral flexibility task (Supplementary Table S7; for additional information regarding individual animal performance in all cognitive tasks see Supplementary Fig. S3d).

As previously described for the HPC, we performed an analysis of autophagic- and dendritic growth-related proteins in the mPFC (Supplementary Fig. S8). When animals were grouped by performance in working memory, within group analysis revealed that in younger animals, levels of LC3-II and p62 were similar between GPs and BPs whereas in older BPs there was a significant decrease in the levels of LC3-II ($t(16) = 2.197$, $p = 0.043$, $d = 1.045$) and a significant increase in the levels of p62 ($t(17) = -3.326$, $p = 0.004$, $d = 1.515$), suggesting a lower level of autophagy in older BPs (Supplementary Fig. S8a,b). Also, older animals, but not younger animals, presented a negative correlation between p62 and the performance in reference ($r = -0.504$, $p = 0.028$) and working memory tasks ($r = -0.646$, $p = 0.003$; Supplementary Fig. S8h), but not the behavioral flexibility task (Supplementary Table S9). Regarding neurotrophins and synaptic markers, mPFC BDNF, PSD95 and SYP protein levels were significantly higher in older BPs compared with older GPs (BDNF $t(18) = -2.226$, $p = 0.039$, $d = 0.995$; PSD95 $t(16) = -1.959$, $p = 0.068$, $d = 0.937$; SYP $t(18) = -2.108$, $p = 0.049$, $d = 0.943$) (Supplementary Fig. S8d–f). Also, BDNF levels were not correlated with performance in working memory (Supplementary Fig. S8i), reference memory or behavioral flexibility (Supplementary Table S9) in any age group, and were correlated with PSD95 levels in older animals ($r = 0.671$, $p = 0.002$; Supplementary Fig. S8k) but not younger rats ($r = -0.256$, $p = 0.476$; Supplementary Fig. S8k; for additional information regarding individual animal performance in all cognitive tasks see Supplementary Fig. S3g). This data is in agreement to what we previously described for the HPC, further showing that the levels of autophagy in mPFC neurons are strongly and positively related with dendritic pruning and better performances in older individuals.

Discussion

The present study addressed the heterogeneity of cognitive aging from a multidimensional perspective to reveal a hitherto of unappreciated complexity. It explored its underpinnings, highlighting, for the first time, the role of the balance between neurotrophic and autophagic activities in such processes. Data herein presented confirmed that, despite a general aging-associated cognitive decline, the performance of both young adult and older rats in working and reference memory tasks is heterogeneous, particularly in older individuals[27]; in the latter, we confirmed that a certain proportion of subjects maintain spatial memory abilities comparable to those of younger animals[6,7]. Of notice, the data clearly revealed, for the first time, that this age-associated increase in the dispersion of individual performance is not universal, as it was not present in all cognitive dimensions (e.g. the behavioral flexibility task).

The working and reference memory tasks both assess spatial learning and memory, albeit within a different timeframe: while the former is dependent on short-term memory and HPC to mPFC connections[28,29], the latter

7

185

depends on long-term memory and largely on the integrity of the HPC[30]. In contrast, the behavioral flexibility task is memory independent and assesses the ability to adapt to changing circumstances[31], a critical component of executive function, which is a very distinct cognitive ability. In light of this distinction, the observation that aging is accompanied by an increased inter-individual heterogeneity (and a performance decline, on average) in memory-dependent but not executive-function-dependent tasks adds yet another layer of heterogeneity to the aging process, and strongly suggests that some cognitive functions (and the networks sub-serving them) are more prone to aging than others. Significantly, this seems also to be the case in humans[32] in which both long-term and working memory are more influenced by age-related impairments than knowledge of vocabulary and priming, a form of non-declarative memory. Despite this, the herein reported relative preservation of executive function in rodents might seem to contradict several studies in humans showing that executive processes are also disrupted in aging[33–35]. However, it is important to highlight that, besides the obvious species difference, executive function tasks in humans are often contaminated by deficits in speed of processing[36], which are well known to be affected by aging. On the contrary, in our experiments, not only was average swimming speed similar in all older subjects (and not significantly different from that of their younger counterparts) but also the behavioral flexibility test is independent of it.

In addition to the two layers of heterogeneity in aging discussed above, analysis of each animal's membership to either the GPs or BPs group, for each behavioral test, further revealed another dimension of inter-individual heterogeneity. Indeed, when comparing cluster membership for each individual in each test, we showed for the first time that most animals were GPs in some tests and BPs in others, without a clear separating pattern in either younger or older groups. Moreover, the overall distribution of animals according to their group membership (GPs or BPs) for the 3 tests (working and reference memory and behavioral flexibility) was strikingly similar in both age groups, with only 13% of older and 19% of younger animals being good in every task, and 22% of older and 19% of younger animals being bad in every task. More importantly, this third heterogeneity level seems to be independent of the other two. In other words, despite all the inter-individual heterogeneity (within a given test) and the heterogeneity between tests (with reference and working memory being more sensitive to aging than behavioral flexibility), there is also heterogeneity at the individual level, as to being a GP or BP for each behavioral test.

In summary, the behavioral data suggest that cognitive decline in aging is not inevitable, or strictly linked to chronological age and that, even in a relatively homogeneous population of animals such as the one in this study, there is a high variability and complexity in the way the different cognitive functions are preserved/impaired in each individual. Understanding the underpinnings of individual differences may help to explain the observed heterogeneity and, possibly, what determines the existence of healthier agers, which was next studied at the morphological and molecular levels.

Changes in the morphology of neuronal dendritic trees were shown to correlate with cognitive alterations in both rodents and humans[57,58]. In aging, it is already well established that memory impairments are not related with neuronal loss[27,39], but rather to volume changes[37] and altered morphology of neuronal dendritic trees[40,41]. In the present work, using a large number of rats (15 young and 27 old) we showed that, on average, older animals have shorter apical dendritic arborizations in dorsal HPC neurons (DG granules and CA1 and CA3 pyramids) but similar apical dendritic trees in mPFC neurons (Cg/PL and IL layers II/III pyramids) when compared to younger animals. These findings are in line with most previous studies that analyzed one or the other region (HPC[42–47]; mPFC[48–50]) and might suggest that the frontal regions might be less affected by the aging process or that age-related changes in neuronal morphology appear later in the mPFC. This is partly corroborated by the fact that age-related apical dendritic retraction in the mPFC was only reported in one study[51]. Interestingly, this relative mPFC "resilience" might be specific for the superficial layers, since deeper layer V pyramidal neurons, similar to hippocampal cells, exhibit an age-related apical dendritic retraction at 20–22 months[20,67,50]. Importantly, this has been observed in humans in which age-related dendritic retraction was 3 times more prominent in the deep than in the superficial PFC pyramids[52]. The fact that mPFC dendrites are less affected by aging fits perfectly with the behavioral data presented here, pointing to an attenuated age-associated decline of executive functions.

Another major novelty of the present work is the finding that older animals with deficits in HPC-dependent tasks have larger dendritic trees in the HPC than cognitively intact rats of the same age. Some previous papers had already shown that HPC cells from older rats[53] and older humans[54,55] had increased dendritic length, but in none of these studies were subjects cognitively characterized. Interestingly, in the mPFC, despite no overall age-related retraction, there was a similar, albeit with smaller magnitude, association between bigger apical dendritic trees in layer II/III Cg/PL pyramids and worse performance in the working memory (mPFC-dependent) test. This association, in older animals, between larger dendritic trees and poorer cognitive function might be considered contra-intuitive. However, while bigger dendritic trees might mean more connectivity and better neuronal function, it is also well described that the accumulation of "waste" dendritic material and large dendritic trees, for example as a result of impaired autophagy and dendritic pruning deficits, hampers neuronal function and correlates with cognitive deficits, in both humans and animals[23]. Interestingly, in Fragile X syndrome patients, who have impaired dendritic pruning, there is also an inverse correlation between HPC volume and cognitive performance, which is not present in age-matched individuals without pruning deficits[56]. Of note, in the present work, the inverse correlations between dendritic tree length and cognitive performance are not present in the group of younger adults (in which an opposite trend is observed), supporting an age-related phenomenon. Together, these results suggest that the inverse correlation between large dendritic trees and poor cognitive performance in the elderly might be attributed to age-associated dendritic pruning deficits, leading to larger, less efficient, dendritic trees. In light of this hypothesis, individual differences in dendritic pruning might also underpin the individual heterogeneity in cognitive aging.

Dendritic pruning is a mechanism often used to selectively remove unnecessary and exuberant neuronal branches, not only in the immature nervous system[23], but also in the adult HPC[57], thus ensuring the

186

proper formation of functional optimized circuitries. Dendritic and synaptic pruning is highly dependent on autophagy-dependent protein turnover, as animals presenting constitutional[23] or induced[58] inhibition of autophagy have larger dendritic trees and increased spine density, which correlate with cognitive deficits. In order to further dissect whether this could contribute to the observed morphology, we analyzed the levels of autophagic activity in the HPC and mPFC. Furthermore, this was complemented with a quantification of the neurotrophin BDNF, a main inducer of dendritic and spine growth[59]. Finally, given the technical challenge to assess protein levels and dendritic tree length in the same region of the same animals, these results were correlated with pre- and post-synaptic markers. Indeed, there is a consensus in the literature that these levels, particularly when concordant, are a good surrogate of synaptic abundance and dendritic tree complexity[60-62]. In support of this assumption, here we show that older, but not young, cognitively impaired animals have higher levels of these synaptic proteins than age-matched cognitively intact rats, precisely replicating the findings from the dendritic tree analysis.

With the present work, we reveal that older cognitively impaired animals have reduced autophagic activity in both the dorsal HPC and the mPFC, when compared with older cognitively intact rats. Of notice is the fact that a decrease in the relative abundance of the autophagic vacuole marker LC3-II (lipidated LC3) was accompanied by a correspondent increase in the relative abundance of the autophagy cargo-protein p62[63], attesting the robustness of the findings. Significantly, these observations were specific for older animals, as levels of autophagic markers in both brain regions did not differ between cognitively intact and cognitively impaired younger individuals. In most organisms, pathological aging is associated with decreased autophagic activity and autophagy inhibition induces degenerative changes that resemble those associated with aging[64]. While the mechanisms of such relationship are far from being well understood, the most prevalent hypothesis considers a failure to clean toxic/waste protein debris, that accumulate with time and induce cellular dysfunction[64]. In line with this, we found that, in the older, decreased levels of autophagy are strongly and inversely correlated with the abundance of synaptic markers, a surrogate marker of dendritic length. Our findings, however, further extend the interpretation of the previous observations, by suggesting that in neurons, decreased autophagy results in less dendritic pruning and an accumulation of dendrites that hamper neuronal function. Significantly, this does not seem to be an inevitable consequence of aging, as levels of autophagic and synaptic markers in older cognitively intact individuals were strikingly similar to those of younger animals. Of note, BDNF levels similarly did not vary significantly with aging but were also increased in older cognitively impaired, compared with cognitively intact animals, suggesting that increased dendritic growth, as well as decreased autophagic activity, might also contribute to the increased dendritic length observed in these animals.

Many factors could induce a decreased autophagic activity, similar to that presented by older cognitively impaired individuals. One of the best candidates is an enhanced activity of the mechanistic target of rapamycin (mTOR, formerly known as mammalian target of rapamycin mTOR) complex, a redox/energy/nutrient sensor that inhibits autophagy and stimulates protein synthesis[65]. Increased mTOR activity (resulting in decreased autophagic activity) has been linked to cognitive dysfunction and learning deficits in a variety of disorders[66,67], which are also associated with an increase in dendritic spines[68]. More importantly, and in line with our results, lifelong treatment of mice[69,70] or accelerated senescence rats[71] with the mTOR inhibitor rapamycin (that is considered an autophagy inducer) improved age-related cognitive dysfunction. Given the above, it is plausible to conclude that an impairment of neuronal autophagic activity could result in a scarcity of pruning mechanisms in aging neural circuits, leading to an accumulation of dendritic material and to the consequent decrease of cognitive performance.

Other factors that are commonly associated with aging could also impact dendritic length, including altered glutamatergic transmission and insulin signaling. However, since these would ultimately lead to changes in neurotrophins and/or autophagic processes, we did not address these separately in the present work. Nevertheless, in order to gain full insight into the individual determinants of altered autophagic activity, these and other factors should be taken in consideration. Furthermore, insights into the relevance of all these mechanisms can only be obtained by experimental manipulations of autophagy, which were not the scope of the present work but should be pursued in the future.

In conclusion, our findings show that alterations in the dendritic length of neurons are associated with the heterogeneity observed in the performance of young and older animals, with a twist. Indeed, it seems that while for younger animals "bigger is better", for older animals "smaller is definitely better". Moreover, we herein provide evidence that, in older animals, dendritic length differences and behavioral heterogeneity can be ascribed to variations in neurotrophin levels and, more importantly, autophagic activity. To summarize these associations, we propose a model where the balance between neurotrophins and autophagic activity regulates dendritic growth/pruning thus contributing to the heterogeneity in the cognitive function of younger vs older animals (Fig. 4). This data represents a paradigm shift in understanding the individual differences observed with aging.

Methods

Animals.   All procedures were carried out in accordance with local regulations (Decreto-Lei no. 113/2013) and European Union Directive 2010/63/EU on animal care and experimentation. Animal facilities and the people directly involved in animal experiments were certified by the Portuguese regulatory entity – DGAV (Direção-Geral de Alimentação e Veterinária). All protocols were approved by the Ethics Committee of the Life and Health Sciences Research Institute (ICVS). All the male Wistar Han rats (Charles River Laboratories, Barcelona, Spain) used in the study were housed in groups of 2 and maintained under standard laboratory conditions: artificial 12 h light/dark cycle (lights on from 08:00 a.m. to 08:00 p.m.); room temperature 22 °C; *ad libitum* access to food and water. A total of 176 old (22–24 month-old) and 102 younger (4–6 month-old) male rats were used in the study. The animals were tested in a battery of water-maze based tests to assess cognition. The brains of a randomly selected subset of both younger and older animals were subjected to morphological (3D neuron reconstruction) analyses and of another randomly selected subset to molecular analyses (western blot). The remainder animals
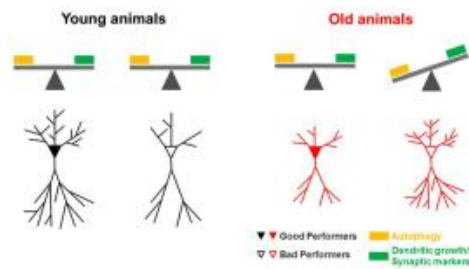
187

Figure 4. Schematic representation of the relations between age, cognitive performance, neuronal morphology, autophagy, synaptic and dendritic growth markers, in the HPC and mPFC. In younger animals "bigger is better"; GPs have the biggest dendritic trees. However, there are no extensive differences in autophagy levels (LC3-II, p62), dendritic growth (BDNF) and synaptic markers (PSD95, SNAP25, SYP). In older animals, it seems that "smaller is better". BPs have the bigger dendritic trees, associated with a decrease in the levels of autophagy (LC3-II, p62), and an increase in dendritic growth (BDNF) and synaptic markers (PSD95, SNAP25, SYP).

were sacrificed at various time points for several other analyses not included in the present study. All behavioral testing was conducted during the light phase of the daily light cycle.

Behavioral assessment. The cognitive status of all the animals was assessed based on performance in a series of tasks using the water maze. Animals were tested during 8 days in 3 tests designed to assess different cognitive domains: spatial working memory, reference memory and behavioral flexibility[72]. The apparatus consisted of a large circular black pool (170 cm diameter), filled to a depth of 31 cm with water (at 22 °C), which was divided by imaginary lines in 4 equal-sized quadrants. During the execution of the test, a submerged cylindrical black platform (12 cm diameter, 30 cm high) was hidden below the water surface at the center of one of the quadrants. The room was dimly lit and extrinsic visual clues were glued to the walls surrounding the tank and kept unaltered during the duration of the experiment. Data was collected using a video camera placed above the center of the pool connected to a video-tracking system (Veiwpoint, Champagne au Mont d'Or, France).

Working memory task. This task is a variation of the spatial reference memory test[20] and depends on mPFC function[28]. Its goal is to assess the ability of rats to learn the position of a hidden platform and to keep this information online during 4 consecutive trials. This test consisted of 4 days of acquisition in which the position of the platform was kept constant during each daily trial (with a maximum of 120 s per trial) but was altered to a different quadrant every changing day (such that all four quadrants were used). Thus, the animal cannot know where the platform was hidden on trial 1 of each day. Test sessions begun with rats facing the wall of the maze, being placed at one of the four different starting points (north, east, south or west) which were different every day. A trial was considered complete once the escape platform had been reached by the rat. The time spent to reach the platform was recorded. Animals were then allowed to spend 30 s in the platform, after which they were towel-dried and allowed to rest in a holding cage some s before being returned to the maze. When the escape platform was not reached within 120 s, the experimenter guided the animal to the platform and an escape latency of 120 s was recorded.

Reference memory task. This task is a HPC-dependent task[30] that evaluates the ability of the animal to learn the location of a hidden platform during 4 consecutive days – spatial reference memory. After the working memory procedure (e.g. on days 5–7), the platform remained in the same quadrant as on day 4 and animals were tested for an additional 3 days without changing the location of the hidden platform. The remaining procedures were like those described above.

Behavioral flexibility task. This is a mPFC-dependent task and was performed after the reference memory task (day 8). For this test, the escape platform was moved and located in the opposite quadrant of where it had been for the previous 4 days. All the procedures were similar to those described above. For this task, time spent swimming in each quadrant was recorded and analyzed.

Histological procedures. Two months after the behavioral evaluation, 50 older (22–24 month-old) and 27 younger (4–6 month-old) rats were randomly selected, deeply anesthetized with sodium pentobarbital and perfused with saline for Golgi-Cox staining (older n = 30, younger n = 15) and western blot analysis (older n = 20, younger n = 12).

188

Golgi-cox staining. After perfusion, the brains were removed, immersed in 25 mL of Golgi-Cox solution[73] (1:1 solution of 5% potassium dichromate and 5% mercury chloride diluted 4:10 with 5% potassium chromate) and kept in the dark at room temperature for 14 days. Brains were then transferred to a 30% sucrose solution. At this moment, brains were stored in the dark at 4 °C from a minimum of 3 days to a maximum of 2 months, before being cut on a vibratome. Coronal sections (200 μm thick) were collected in 6% sucrose and blotted dry onto cleaned, gelatin-coated microscope slides. Subsequently, sections were alkalinized in 18.7% ammonia, developed in Dektol (Kodak), fixed in Kodak Rapid Fix, dehydrated through a graded series of ethanol of increasing concentrations and cleared in xylene before being covered in mounting media (Entellan New) and coverslipped. The slides were stored in the dark and exposed to the air, at room temperature, until being analyzed.

Structural analysis. To ensure an unbiased analysis, slides were re-coded by the lab technician (not involved in the research) as soon as they were prepared and all neuronal reconstructions were done blind to animal age or performance group. To minimize bias, codes were only broken after all data was collected and entered into the database.

Dendritic tree analysis. Dendritic arborizations were analyzed in the DG, CA3 and CA1 regions of the dorsal HPC, and in layer II/III of the Cg/PL and IL areas of the mPFC. The dorsal/ventral HPC division was performed according to Pinto et al.[74] and the identification of layer II/III of the Cg/PL and IL areas was achieved according to Cerqueira et al. (2007b)[75].

The granular neurons of the DG were readily identified based on their round cell bodies, which are located in the stratum granulosum of the suprapyramidal and infrapyramidal blades. Pyramidal neurons from the HPC (CA1 and CA3) or the mPFC (Cg/PL and IL) were readily identified by their characteristic triangular shaped soma. All neurons were chosen for reconstruction based on the criteria described by Uylings et al.[76]: (i) full impregnation of the neurons along the entire length of the dendritic tree; (ii) apical dendrite without truncated branches, except on the most superficial layer; (iii) presence of at least 3 primary basal dendritic shafts, each of which branched at least once (when applicable); (iv) relative isolation from neighboring impregnated cells that could interfere with analysis (clear somatic boundaries) (v) no morphological changes attributable to incomplete dendritic impregnation of Golgi-Cox staining. To minimize selection bias, slices containing the region of interest were randomly searched and the first 5–10 neurons fulfilling the above criteria (maximum of 3 neurons per slice) were chosen. For each selected neuron, all branches of the dendritic tree were reconstructed at 600× magnification, using a motorized microscope (Olympus BX51 Microscope with oil-objectives), attached to a camera (QImaging® Retiga-2000R digital camera, Surrey, Canada) and the Neurolucida software (Microbrightfield, VT, USA). A 3-D analysis of the reconstructed neurons was performed using NeuroExplorer software (Microbrightfield). Dendritic morphology was examined by assessing the total dendritic length and the number of dendritic branches. In addition, to assess differences in the arrangement of dendritic material, a 3-D version of a Sholl analysis[77] was performed. For this, the number of intersections of dendrites with concentric spheres positioned at radial intervals of 20 μm from the soma was recorded.

Western blot analysis. Rat brain tissue (dorsal HPC and mPFC) was lysed with 1X RIPA buffer supplemented with protease inhibitors (cOmplete Mini EDTA-free, Roche, Basel, Switzerland) and phosphatase inhibitors (phosphatase inhibitor Cocktail 2 and 3, Sigma-Aldrich, St. Louis, Missouri, US). Homogenization was performed with an electric homogenizer, and homogenates were maintained in constant agitation for 2 h at 4 °C. After that, homogenates were centrifuged at 12000 rpm at 4 °C for 20 min and supernatants were collected for western blotting. Protein concentration was determined using the Bradford assay (Bio-Rad, Hercules, CA, USA). Twenty micrograms of total protein were loaded into SDS-Page gels and then transferred to nitrocellulose membranes. The membrane was stained with Ponceau to verify successful transfer. Membranes were incubated with the following primary antibodies: LC3 (1:1000, Cell signaling, Danvers, US), p62 (1:1000, Novus, St. Charles, US), BDNF (1:1000, Abcam, Cambridge, UK), SNAP25 (1:5000, Abcam), SYP (1:10000, Abcam), PSD95 (1:1000, Abcam), α-tubulin (1:5000, Sigma-Aldrich, St. Louis, Missouri, US), and β-actin (1:1000, Ambion, Naugatuck, US), overnight at 4 °C. Antibody affinity was detected by chemiluminescence (ECL Bio-Rad). Band quantification was done using ImageLab 4.1 (Bio-Rad) using α-tubulin or β-actin as the loading control. Full-length images are presented in Supplementary Fig. S10.

Statistical analysis. All statistical analysis was conducted in the SPSS software package version 19 (IBM corporation, Armonk, New York, US). After confirmation of homogeneity and normality, appropriate statistical tests were applied to the data. To facilitate direct comparisons between different tests, results of all behavioral tests were converted to a 0–100% scale, where 0% indicates worst possible performance (120 s to reach the platform for the working and reference memory tests or no time in target quadrant for the behavioral flexibility task) and 100% indicates best possible performance (0 s to reach the platform or total time in the target quadrant). Also, in order to allow individual correlations between the performance of the animals in the working and reference memory tests, which consist of several testing days, and structural parameters, a performance index (PI) was calculated for each test by employing the following formula: $PI = (P1 + (P3 + P4)/2)/2$ where Pn represents the average performance of each animal on trial n (for working memory) or day n (for reference memory); importantly, the index value can be directly read as the average performance of each animal per trial/day. Regarding the behavioral flexibility test, the percentage of time spent in the target quadrant (performance index) was used to assess the individual correlations. Clustering of animals in good and bad performance groups was done using the k-means cluster analysis according to their performance index in the working memory, reference memory or behavioral flexibility tasks. The distance between cluster centers of the two desired groups was maximized in an iterative process (maximum number of iterations set to 25). Comparisons between two groups were done using

189

the two-tailed t-test and repeated measures ANOVA. Two-way ANOVA was used to evaluate the impact of age and the effect of group performance in further behavioral, structural and molecular data. For multiple comparisons in the behavioral tasks, one-way or repeated measures ANOVA were used. Differences between groups were then determined by Tukey´s honestly significant difference test post-hoc analysis. Pearson correlations were computed between continuous variables. Measures of effect size (Cohen's d, Eta-squared or Pearson correlations) are presented whenever appropriate. Note that, regarding the number of younger animals that performed the behavioral flexibility task, only 101 out of the 102 animals were included in the analysis; this was due to tracking problems. Results are expressed as group mean ± SEM. Differences were considered significant if $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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191

Author Contributions

C.M., J.A.P., J.C.S., N.S. and J.J.C. designed research; C.M., R.T., S.P.N., S.M-M. and S.M. performed research; C.M. analyzed data; and C.M., J.C.S., N.S. and J.J.C. wrote the paper.

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192

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Assunto: | PROTECÇÃO DOS ANIMAIS UTILIZADOS PARA FINS EXPERIMENTAIS E/OU OUTROS FINS CIENTÍFICOS - PEDIDO DE ACTUALIZAÇÃO DE PROJECTO DE EXPERIMENTAÇÃO ANIMAL

Na sequência do pedido de V. Exª no sentido de poder ser autorizada a realização do projecto experimental designado **"Envelhecimento: factores preditivos e moduladores"** e dos esclarecimentos que nos foram prestados relativamente às dúvidas que a sua análise inicial nos suscitou – quer a esta Direcção Geral, quer a alguns membros da Comissão Consultiva prevista na alínea b) do nº 49, da Portaria nº 1005/92, de 23 de Outubro -, os quais muito agradecemos, cabe-me informar que o projecto em apreço poderá ser levado a efeito.

Com os melhores cumprimentos,

O Director de Serviços

As) António Pina Fonseca

APM/