Effects of caloric restriction on brain aging and cognitive decline: Behavioral and biochemical analysis

Divka Inge Rojic Becker

Ph.D. Thesis

Neuroscience

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"Se hacen eternas cuando las quieren
Y siempre viven y nunca mueren
Cuando se duermen son indefensas
Y se despiertan cuando las piensas
Y las atacan y las defienden
Las más valiosas nunca se venden
Alcanzan todo lo que deseas
Así de grande son las ideas".

CONTENTS

ABBREVIATIONS

In alphabetic order

AADC	Aromatic l-amino acid decarboxylase
ALP	Alkaline phosphatase
AMPAr	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
AMPA1	Subunit 1 of AMPAr
AMPA2	Subunit 2 of AMPAr
AMPK	5' AMP-activated protein kinase
ANOVA	One-way analysis of variance
ATP	Adenosine triphosphate
BBB	Blood brain barrier
BDNF	Brain-derived neurotrophic factor
BMI	Body mass index
BN	Brown-Norway strain of rats
BSA	Bovine serum albumin
CA	Cornus ammonius
CALERIE	Comprehensive assessment of long-term effects of reducing calorie intake
CNS	Central nervous system
CR	Caloric restriction
CRM	Caloric restriction mimetics
DA	Dopamine
DG	Dentate gyrus
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferases
DNMTA3a	Subunit 3a of DNA methyltransferases
DOPAC	3,4-Dihydroxyphenylacetic acid
EC	Entorhinal cortex
EPM	Elevated plus maze
FC	Frontal cortex
FL	Frontal lobe
F344	Fischer 344 strain of rats
GABA	Gamma-aminobutyric-acid

GH	Growth hormone
Glu	Glutamate
HDL	High-density lipoprotein
HPA	Hypothalamic-pituitary adrenal axis
HPC	Hippocampus
HPLC	High performance liquid chromatography
HT	Hypothalamus
HVA	Homovanillic acid
IACUCs	Institutional animal care and use committees
IGF-1	Insulin-like growth factor 1
IF	Intermittent fasting
IFN-ɣ	Interferon gamma
L-DOPA	3,4-dihydroxyphenylalanine
LB	Loading buffer
LDL	Low-density lipoproteins
LTD	Long-term depression
LTP	Long-term potentiation
MAO	Monoamine oxidase enzyme
mGluR	Metabotropic glutamate receptor
mPFC	Medial prefrontal cortex
mTOR	target of rapamycin in mammals
MWM	Morris water maze
NA	Noradrenaline
NAD^+	Oxidized nicotinamide adenine dinucleotide
NMDAr	N-methyl-D-aspartate receptor
NMDAR1	Subunit 1 from NMDAr
NMDAR2A	Subunit 2A from NMDAr
NMDAR2B	Subunit 2B from NMDAr
NT	Neurotransmitter
OF	Open field
p-CREB	Phosphorylated cAMP response element-binding protein
PBS	Phosphate-buffered saline

PGC-1α	Transcriptional coactivator peroxisome proliferator-activated receptor gamma coactivator 1 alpha
PFC	Prefrontal cortex
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
SGZ	Subgranular zone
SIRT-1	NAD-dependent deacetylase sirtuin-1
SIRT-2	NAD-dependent deacetylase sirtuin-2
SUB	Subiculum
SYP	Synaptophysin
TH	Tyrosine hydroxylase
TNF-α	Tumor necrosis factor
TPH	Tryptophan hydroxylase
Tryp	Tryptophan
Tyr	L-Tyrosine
ULK1	UNC-51-like kinase 1
V-SVZ	Ventricular-subventricular zone of the lateral ventricle
VTA	Ventral tegmental area
5-HIAA	5-Hydroxyindoleacetic acid
5-HT	5-Hydroxytryptamine/Serotonin
5-HTP	5-Hydroxytryptophan

I. INTRODUCTION

Nowadays one of the major problems developed societies face is the progressive aging of the population. According to data from World Population Prospects, between 2015 and 2030 the number of people in the world over the age of 60 years is expected to grow by 56 per cent. By 2050, the global population of elderly people is predictable to reach nearly 2.1 billion (United Nations, 2015) (Figure 1). This increase will also require higher levels of spending on healthcare and welfare for the elderly. Furthermore, an increase in life expectancy will lead to a higher incidence of age-related diseases that health services will need to be equipped to address. For instance, age related neurodegenerative disorders such as Alzheimer, Parkinson and other types of dementia and cardiovascular pathologies are likely to increase, which will present major social and medical challenges for governments. Therefore, it will become increasingly relevant to understand the cognitive changes that accompany normal and pathologic aging. (World Population Prospects: the 2015 Revision (United Nations, 2015).

Aging, defined as time-dependent functional deterioration that affects most living organisms, is a process that involves physical and cognitive decline characterized by a progressive loss of integrity, impaired function and increased vulnerability to death (López-Otín et al., 2013). Although there is significant heterogeneity among older adults in the rate of cognitive decline, the basic mental functions most affected by age are attention, cognitive flexibility, as well as both short and long-term memory (Bizon et al., 2012). Moreover, these age-related cognitive changes have been shown to be directly associated to mark structural and functional alterations in aging brains. The aged brain comprises a decrease in the grey matter volume and a significant reduction in the number of synapses. In addition, alterations in the pyramidal dendritic spines, a reduction in monoaminergic and glutamatergic transmission, neurovascular changes such as deterioration in the blood brain barrier and an increase in inflammatory processes have also been detected. These changes in the brain correlate with alterations in executive functions and memory consolidation (Izquierdo, 2001). The main brain areas underlying the cognitive age-related decline and those most affected by the aging process are the Hippocampus (HPC) and the Prefrontal cortex (PFC). In fact, experimental studies on cognitive aging in rodents have shown a deterioration of the anatomy and physiology of the HPC (Kuhn et al., 1996) and the PFC (Taylor et al., 2003). Actually, during aging these areas are affected by an increase in cell oxidative stress, neuroinflammation, impaired monoaminergic and glutamatergic transmission and reduced synaptic plasticity, which may impair cognitive functions (Bettio et al., 2017).

Over the last few years, evidence has emerged which suggests that healthy lifestyles may decrease the rate of age related cognitive decline and help delay the onset of cognitive symptoms of age-associated diseases. Activities such as regular physical exercise (García-Mesa et al., 2015), cognitive stimulation, intermittent fasting (IF), and caloric restricted diets may slow down the progression of age-dependent diseases and may be one of the keys to an active and satisfactory senescence (Phillips 2017). The importance of a healthy, balanced and nutritious diet as a protecting factor against aging has gain particular relevance in the last decade. For example, the typical diet of the people of Okinawa, a Japanese island, has become popular due to the fact that the average life expectancy is higher than in the continental area (Willcox et al., 2007). Their diet consist of different green leafy vegetables and a small amount of animal protein, namely fish (Redman et al., 2008). Remarkably, this diet has an optimum content of dietary protein and carbohydrates, and is almost identical to the diet administered to laboratory animals nowadays (Le Couteur et al., 2016).

In this context, one of the most common procedures to delay age-related cognitive decline is the caloric restriction (CR) diet. It is considered to be one of the most effective dietary interventions to increase longevity and improve health during aging (Gillespie et al., 2016). CR is defined as a reduction in the intake of calories without causing malnutrition and with a normal consumption of vitamins, minerals and essential biomolecules (Ribarič, 2012). Around one century ago, it was observed for the first time that a reduction in food intake improved life expectancy in laboratory animals (Osborne et al., 1917). Since then, a CR diet has been given to laboratory animals to be used as a robust research paradigm in aging studies in rodents (Minor et al., 2010). This model has verified in different animal species, from invertebrate to vertebrate beings (Fontana & Partridge, 2015). Even studies in non-human primates show that said dietary intervention noticeably improves the health of primates (Balasubramanian et al., 2016). In humans, the application of CR is controversial and difficult due to the struggle associated with maintaining the diet for long periods of time as well as moral and ethical issues (Arslan-Ergul et al., 2013). That is why the development of pharmaceutical CR mimetics, which mimic the same hormonal and physiological effects of CR, may be the future in this field (Ingram et al., 2006).

Nevertheless, while the consequences of CR for general health are well determined, its ability to slow the cognitive decline that accompanies aging remains controversial. Consequently, the aim of the present study was to determine the effects of life-long CR on learning as well as short and long-term memory processes, and also analyze specific biochemical markers in the brains of aged rats. By achieving these objectives, we hope to gain a more specific and determined insight into the relationship between diet and mental health; and therefore, contribute to the

search for strategies to promote a healthy longevity which will have a significant impact on improving the quality of life of millions of older adults in the world.

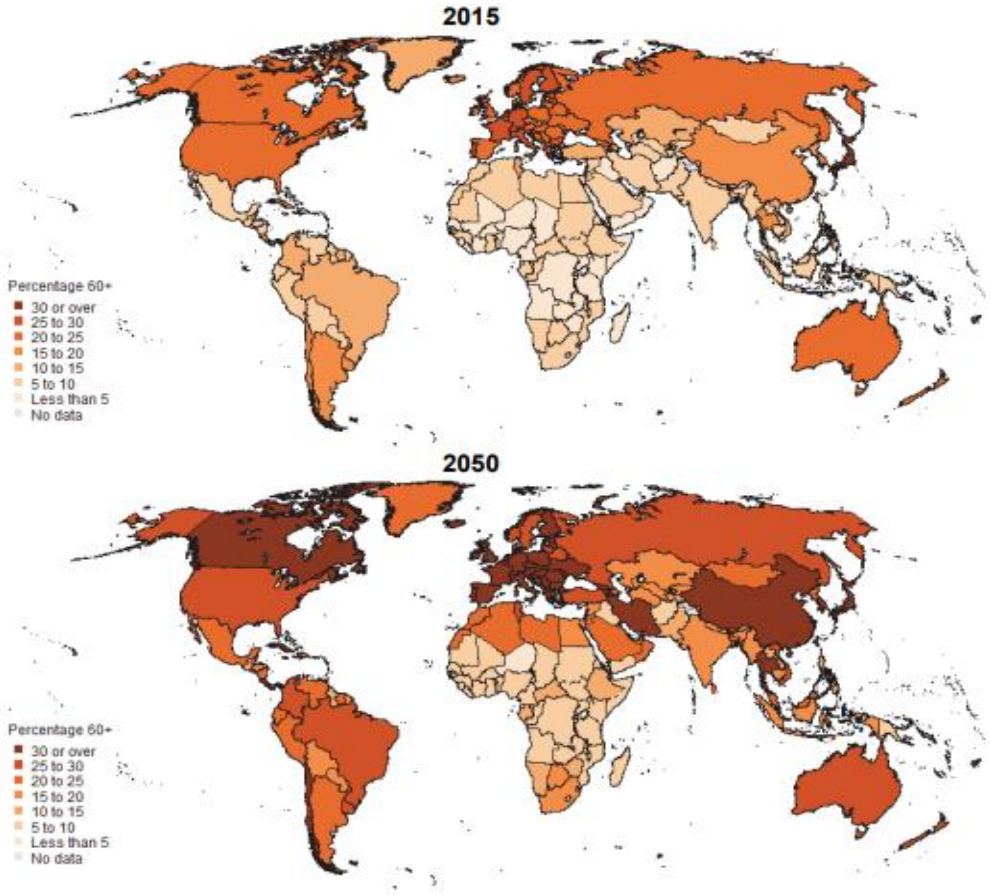

Figure 1: Map of percentage of population aged 60 and over in 2015 and 2050. Data source: United nation (2015) World population prospect: The 2015 revision.

II. APPROACH AND OBJECTIVES

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The increase in the average life expectancy of people in developed societies has generated new scientific challenges in the search for procedures to enhance the quality of life of the elderly. The aging process affects the physical and cognitive functions of all animals. The central nervous system (CNS) specifically shows clear signs of aging at a macroscopic level; whereas the volume and weight of white matter decreases in a diffuse and uniform manner throughout the brain (Liu et al., 2017), grey matter is affected specifically in the parietal and frontal cortices, as well as other structures such as the striatal nuclei and the HPC (Shankar, 2010). At a molecular level, CNS aging in numerous species of mammals shares common characteristics such as synaptic atrophy, abnormalities in the cytoskeleton, an increase in the reactivity of astrocytes and microglia (Prolla & Mattson, 2001; Shankar, 2010), synaptic alterations of the pyramidal neurons of the HPC (Morrison & Baxter, 2012), and a decrease of monoaminergic transmission in regions such as PFC, striatum and HPC (Stemmelin et al., 2000). Some studies (Yamamoto et al., 1991), have also shown a decline in certain peripheral hormones such as type 1 Insulin-like Growth Factor (IGF-1), a hormone that protects against type 2 diabetes and osteoporosis (Barzilai & Bartke, 2009), and glucocorticoids (Yau & Seckl, 2012), hormones associated with the stress response. All these neurobiological alterations are the main underlying processes that affect the cognitive decline during aging. In addition, the mental functions most affected by aging are attention and memory. The learning tasks showing the highest levels of deterioration are those that require a flexible control of attention and working memory, both cognitive functions associated with the Frontal lobe (FL) (Bizon et al., 2012) and those associated with the HPC and adjacent structures of the medial temporal lobe (Robitsek et al ., 2008). Therefore, the animal models used to assess the age-dependent cognitive deficit have basically focused on spatial learning tasks that are dependent on this specific brain area.

Age-dependent cognitive decline and the incidence of neurodegenerative diseases constitute major social and health problems nowadays. The attempt to develop guidelines to achieve healthy aging and reduce cognitive impairment has led researchers to study how certain habits can modulate the brain mechanisms involved in mental deterioration. One such example is the effects of dietary interventions such as IF and CR; both have been shown to reduce age-related functional impairment and therefore to improve well-being during aging (Gillespie et al., 2016). In terms of health, the benefits of CR have been well determined and seem to derive from its capacity to reduce metabolic rate, oxygen consumption and blood glucose levels and to contribute to an improvement in the function of the immune system (Prolla & Mattson, 2001).

Moreover, CR has been shown to be a very effective procedure in reducing the mortality of laboratory animals, enabling brain aging studies (Goto et al., 2007; Mattson et al., 2003). The results of research on this topic have shown that CR reduces the expression of genes associated with inflammatory response and increases the expression of neurotrophins, such as Brain derived neurotrophic factor (BDNF), as well as promoting neurogenesis in the dentate gyrus of the HPC (Park & Lee, 2011). It has also been observed that CR is capable of reducing the adverse effects of aging on glutamatergic transmission in the cerebellum, cortex and HPC (Monti et al., 2004). In fact, glutamate (Glu) is the most relevant excitatory neurotransmitter (NT) in the CNS and it has been widely linked to memory formation and synaptic plasticity mechanisms such as long-term potentiation (LTP) and depression (LTD) (Molinari et al., 2012). Along similar lines, substantial amounts of data indicate that LTP is induced by the activation of the glutamatergic receptors N-methyl-D-aspartate (NMDAr) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPAr) (Martin et al., 2000), both receptors affected by the aging process. Moreover, the dietary intervention has been able to enhance learning and memory in aged rats (Geng et al., 2007; Gyger et al., 1992) and in mice (Bellush et al., 1996; Dong et al., 2016; Kuhla et al., 2013).

Although the experimental background exposed to date would suggest that CR might be a good procedure to delay age-related cognitive decline, the issue has scarcely been examined and the few available results are rather contradictory. The investigation of the effects of CR in laboratory animals has provided the first advances in the understanding of its beneficial effects. While some studies with ageing animals have shown that CR may improve learning and memory (Carter et al., 2009; Fitting et al., 2008; Markowska & Savonenko, 2002), others have reported no beneficial effects of CR on mental functions (Beaty et al., 1987; Stewart et al., 1989). The discrepancies observed may be due to the use of animals of different strains and ages, the types of CR chosen and the learning models assessed, as they have been based exclusively on spatial tasks. Therefore, the capacity of CR to improve cognitive capacity during aging continues to be a topic of debate that requires further study. Consequently, this doctoral thesis aims to investigate the potential beneficial effects of CR on the nervous system during aging and, specifically, on cognitive function. For these purposes, in the present study we analyzed the effects of CR in old rats (24 months) to test whether this diet is effective in preventing age-associated deficits. Thus, aged animals with CR, aged animals with unrestricted access to food (*Ad Libitum*) and adult rats were trained in different behavioral tasks related to brain areas such as the HPC, the PFC and striatal nuclei, afterwards both short and long-term memory were assessed. The neurophysiological mechanisms underlying the beneficial effects of CR on learning and memory

were studied by analyzing the expression of Glu receptors and synaptophysin (SYP), a presynaptic membrane protein essential for neurotransmission, in those brain areas. In addition, brain monoamines levels and plasma levels of hormones, such as corticosterone, insulin, leptin and IGF-1 were examined and correlated with the effects of CR on learning and memory processes. Where this study differs from past studies lies in the fact that all such variables will be analyzed in rats of the same strain (Wistar) and age, kept in the same environmental conditions, with the only difference being their life-time diets.

The hypothesis of the present research was that brain aging might contribute to age-dependent cognitive decline; therefore, dietary interventions such as CR, which diminish the effects of aging on the nervous system, could be a strategy for improving learning and memory. Specifically, CR may reduce cognitive decline due to aging by acting on neurophysiological mechanisms such as:

a) The monoaminergic neurotransmission of brain regions related to cognitive processes such as the HPC, the frontal cortex (FC), and the striatum.
b) The glutamatergic receptors and proteins involved in synaptic plasticity,
c) The plasma hormone levels such as corticosterone, insulin, leptin and IGF-1.

The specific research objectives in this research are the following:

1) To compare the learning and memory capacity of aged animals on the basis of the type of diet administered, CR vs. *Ad Libitum*, and young animals in several tasks:

a) Morris water maze (MWM), a relational memory task that is hippocampal-dependent.

b) Odor discrimination task (ODT), a simple odor-reward association task that depends on the FC.

c) Object recognition memory in a Y maze, a prefrontal and hippocampal dependent memory task.

2) To verify whether emotional and motor variables (using open field and elevated plus) of the aged animals may have affected cognitive outcomes.

3) To assess the effects of CR vs. *Ad Libitum* on the animals' general health, comparing biochemical measures obtained from blood plasma: levels of triglycerides, cholesterol, alkaline phosphatase (ALP), calcium, albumin and glucose.

4) To analyze the plasma levels of hormones such as insulin, leptin, corticosterone and IFG-I of aged rats in both food environments and young animals, as this may have an influence on the animals' cognitive capacity.

5) To determine whether ageing in CR vs. *Ad Libitum* conditions favors monoaminergic transmission (quantification of the levels of dopamine (DA), noradrenaline (NA) and serotonin (5-HT) and some of their metabolites) in the HPC, striatum and FC using High Performance Liquid Chromatography (HPLC).

6) To verify whether ageing in CR vs. *Ad Libitum* conditions favors glutamatergic transmission (quantification of ionotropic receptors NMDA and AMPA), tyrosine hydroxylase (TH) and tryptophan hydroxylase (TPH) and SYP, a protein associated with synaptic plasticity in the HPC, striatum and the FC by means of Western Blot (WB).

III. THEORETICAL FRAMEWORK AND EXPERIMENTAL BACKGROUND

III. THEORETICAL FRAMEWORK AND EXPERIMENTAL BACKGROUND

1. Cerebral aging and cognitive impairment

Aging is a complex biological process associated with declines in sensory, motor, and cognitive functions. However, aging is not a disease, it is a normal physiological process that can develop without the appearance of concurrent diseases (Mora, 2013). Aging also refers to a physiological process that occurs in most species and denotes the loss of the ability to adapt to any change in the environment, a necessary ability to be able to relate to each other satisfactorily (Izquierdo, 2001). Moreover, the cerebral changes that happen during aging may have an impact on cognitive processes in domains such as executive functions, processing speed and memory, which start to deteriorate in the third decade of life in humans (Salthouse, 2012).

1.1 The aging brain

1.1.1 Volume and structural changes

One of the most relevant age-related structural changes is the 16-20% decrease in white matter volume that has been shown to occur in those over the age of 70 compared to younger people (Meier-Ruge et al., 1992). This is particularly significant in the precentral gyrus, gyrus rectus and the corpus callosum, areas which do not experience a substantial decline in grey matter volume during aging. In addition, a reduction in parahippocampal white matter, which is related to lower communication within the hippocampal structures, has also been detected in old people. This decrease in white matter is one of the main factors that has been most associated with age-dependent memory decline (Kennedy & Raz, 2009).

The volume of grey matter starts to shrink around the age of 20 (Terry & Katzman, 2001), but this atrophy is especially prevalent in the PFC (Peters, 2006), whereas in the temporal cortex, in areas such as the HPC, this reduction is more moderate. Moreover, an inverse relationship between age and prefrontal cortical grey matter volume has been observed in humans (Terribilli et al., 2011). The loss of grey matter is mainly attributed to decreases in the size and number of connections between neurons. This is particularly evident in the decline in the complexity of the dendrite arborization or in the neuritic spines, which leads to a decrease in synaptic density in hippocampal areas (Dickstein et al., 2007). One of the reasons for the drop in HPC and PFC volume may be neuronal loss. However, a complementary process that may also contribute to it is a potential decline in neuronal production or neurogenesis with age (Bettio et al., 2017).

Neurogenesis, the process of producing new neurons over the course of life, happens in many areas of the brain, but especially in the sub-granular zone (SGZ) of the dentate gyrus (DG). These new neurons, generated in the SGZ, migrate to the DG granule cell layer, where they undergo dendritic arborization into existing neural circuitries, and then contribute to the function of the HPC (for a review see Bettio et al., 2017). Previous findings have revealed that an adult human HPC is able to generate around 700 new neurons per day (Spalding et al., 2013), while in rodents the number increases to 9000 new neurons per day (Cameron & Mckay, 2001). However, a recent study (Sorrells et al., 2018) in humans and non-human primates puts into question the previous results about neurogenesis. The experiment demonstrated that the recruitment of young neurons to the HPC decreases rapidly during the first few years. In addition, the findings in humans reveals that neurogenesis in the DG is either extremely rare or does not continue during adulthood. In addition, controversial results have been found about the replacement of neurons during adulthood and aging. Some studies indicated that it is not really affected by the aging process (Spalding et al., 2013) or it is downregulated (Dennis et al., 2016). Despite these discrepancies, it is generally believed that the adult human HPC continues to generate new neurons; however, this process either decreases or is non-existent during aging. These debatable results seem only to affect humans and primates, since there are no previous experiments that corroborate the lack of neurogenesis in other mammals and especially not in aged rodents. Further investigation is required to get the bottom of these contradictions.

The underlying processes that may affect the reduction in neurogenesis in the aged HPC may be a result of an increase in the levels of pro-inflammatory cytokines, and/or a reduction in the release of growth factors by hippocampal astrocytes (for a review Bettio et al., 2017). An aging organism is also subjected to generalized metabolic changes that may also affect neurogenesis. The aging process produces a general decrease in mitotic activity in the brain (Georg Kuhn et al., 1996), as well as a drop in glucose consumption in the HPC and PFC (Gage et al., 1984), both factors linked with the downregulation of neurogenesis. Furthermore, the decrease in HPC volume and the diminution of hippocampal neurogenesis seems to affect cognitive processes such as memory consolidation and emotional behavior (Anacker & Hen, 2017), which may be linked to age-related cognitive decline.

1.1.2 Vascular alterations

During aging, neurovascular changes and alterations in inflammatory processes may contribute to cognitive decline. Neurovascular alterations, defined as changes in cerebral capillaries and glial vascular degeneration, seem to be related to the age-related memory loss observed in

animals (Zhang et al., 2012). These changes indicates that memory deterioration may be linked to the early onset of neural dysfunctions such as neurovascular alteration, prior to the well-known neuronal degeneration (Zhang et al., 2012). Furthermore, the age-related breakdown of the blood brain barrier (BBB), a semipermeable membrane that separates circulating blood from the brain and the extracellular fluid in the CNS, allows neurotoxic proteins to enter the CNS, which can result in a loss of brain homeostasis thus causing oxidative stress, cell death and neuroinflammation (Enciu et al., 2013). This process is the reaction of the glial cell, particularly astrocytes and microglia, to brain infections, neurodegenerative processes or prion diseases. When neuroinflammation becomes chronic, it might contribute to neuronal dysfunction and cell loss (Streit et al., 2004).

The breakdown of the BBB has been observed in humans and seems to be an initial step of human brain aging that may contribute to cognitive decline (Montagne et al., 2015). Moreover, the basement membrane (a component of the BBB) shows signs of deterioration and shrinkage in the aged brain. This degeneration can lead to vascular leakage and astrocyte dysfunction in mice, which strongly correlates with an increase in neuroinflammation and neuronal dysfunction (Soto et al., 2015). In addition, the HPC is the first structure to suffer the breakdown of the BBB. This process may happen because the HPC is more vulnerable to experiencing an increase in glucocorticoid concentration, oxidative stress, neuroinflammation and the accumulation of amyloid-β peptide aggregates, which would explain endothelial dysfunction (Bettio et al., 2017).

1.1.3. Functional changes in synaptic proteins

In general, an aged brain displays a decrease in the number of synapses, an alteration of the dendritic spines leading to dendritic atrophy and the dysfunction and reduction of the expression of multiple synaptic proteins such as Glu receptors and SYP. Glu is the main, most prominent excitatory neurotransmitter of the human CNS (Molinari., 2012). It is also the precursor for *gamma*-Aminobutyric acid (GABA), the main inhibitory neurotransmitter. Among other proteins, Glu receptors are critically involved in synaptic plasticity. There are two types of Glu receptors: ionotropic and metabotropic. The ionotropic receptors are associated with ion channels that rapidly trigger electrical activation. There are four types of ionotropic Glu receptors: NMDAr, AMPAr, kainate and delta receptors. These receptors are expressed all over the brain, but mostly in areas such as the HPC, the amygdala, the striatum and the PFC. These receptors are ligand-gated nonselective cation channels for K^+, Na^+ and Ca^{2+} in response to glutamate binding, causing excitatory postsynaptic current. The flow of Ca^{2+} through NMDAr is thought to cause both LTP and LTD by transducing signaling cascades and regulating gene

expression (Barbado et al., 2009). AMPAr main functions are critical for nearly all aspects of brain function, including learning, memory, and cognition (Henley & Wilkinson, 2013). In addition, ionotropic receptors are considered to be an interesting target for enhancing cognitive functions due to their fundamental role in learning, memory encoding, LTP and LTD (Morris, 2013). It has been observed that the density of NMDAr and AMPAr is relevant for the regulation of synaptic plasticity and for learning and memory processes (Clayton et al., 2002). NMDAr and AMPAr are composed of various subunits. The postsynaptic effects in response of Glu will depend on the subunit that is triggered. For this investigation, the action of the NMDAR1 and NMDAR2A subunit are interesting due to their role in synaptic plasticity. These receptors are located mainly in the presynaptic region where they seem to have a Glu modulating function (Rodriguez-Moreno et al., 2011). It is due to its multifunctionality and relationship with memory that it has been considered in research as an interesting target in the enhancement of cognitive functions (Collingridge et al., 2013). Moreover, the main subunits of AMPAr that will be analyzed in this thesis are AMPA1 and AMPA2, both Glu receptors seem to underlie the effect in synaptic plasticity and memory formation associated with cognitive impairment during aging and neurodegenerative diseases (Henley & Wilkinson, 2013).

During aging there is a general decrease in ionotropic glutamatergic receptors throughout the brain, particularly in the HPC and PFC. Thus, in aged rats, the synapses in the perforant path of the DG appear to be particularly affected, showing a decrease in NMDAr responses by fewer synaptic contacts (Barnes et al., 2000). In particular, an important decrease has been recorded in the subunit NMDAR1 (Eckles-Smith et al., 2000) in the HPC (Clayton et al., 2002). Those receptors are essential for LTP induction and maintenance (Eckles-Smith et al., 2000). Moreover, both NMDAr and AMPAr have been implicated in structural changes associated with synaptic plasticity (Shi et al., 2007), including synapse formation, maintenance and remodeling (Fischer et al., 2000; Lüscher et al., 2000). In addition, several studies (Izquierdo et al., 2008) in rodents have demonstrated that aging is associated with impaired hippocampal LTP. This loss of synaptic plasticity is related with a diminution in the ability to consolidate long-term memory. In general, the deregulation of Glu neurotransmission may be the cause of age-related alterations in hippocampal synaptic transmission (Newton et al., 2008).

The activation of metabotropic Glu receptors (mGluR), which are associated with G proteins, causes said G proteins to bind to the intracellular region to be phosphorylated, affecting multiple biochemical pathways. Because of this, those receptors can increase or decrease the excitability of the postsynaptic cell, causing a wide range of physiological effects (Platt, 2007). These receptors can be found mainly in the HPC, cortex and cerebellum. The metabotropic actions are

longer in duration, but slower in action than ionotropic receptors. The physiological effects of these receptors are mostly molecular adaptations and changes in gen expressions. This is the main reason why they are involved in learning, memory, synaptic plasticity, anxiety and perception of pain (Ohashi et al., 2002).

Another relevant protein that declines during the aging process is SYP. SYP is a presynaptic membrane protein essential for neurotransmission in hippocampal neurons. It is one of the most widely used proteins as a marker of synaptic plasticity. It has been shown, via immunohistochemistry, that old aged rats have a smaller number of synapses and synaptic vesicles and less expression of SYP in the HPC when compared to young animals (Wang et al., 2007). In addition, rodents that showed spatial learning deficits showed a significant reduction in SYP immunoreactivity in the *Cornus Ammonius 3* (CA3) of the HPC in comparison to young control or age matched rats with preserved learning (Smith et al., 2000). This diminution was also observed in *Ad Libitum* male F344x Brown-Norway (BN) hybrid rats, which demonstrated a decrease of SYP in CA3, not in CR animals (Adams et al., 2008).

1.1.4. Monoaminergic transmission

The monoaminergic NT and neuromodulators group, which include catecholamines (dopamine, noradrenaline and adrenaline) and serotonin, are involved in many brain functions that change throughout life. All monoamines are derived from aromatic amino acids such as L-tyrosine (Tyr), phenylalanine, tryptophan (Tryp), and thyroid hormones through the action of the l-amino acid aromatic decarboxylase (AADC) enzyme (Míguez et al., 1999). For instance, 5-hydroxytryptamine (serotonin, 5-HT) and 3,4-dihydroxyphenethylamine (dopamine, DA) are two NTs which are synthesized and released in brain areas such as the HPC and the striatum, closely related to cognitive functions such as learning and memory (González-Burgos & Feria-Velasco, 2008). In general, monoamines exhibit a marked decline in different brain regions as part of normal aging (Míguez et al., 1999). Therefore, this group of NTs might be, at least in part, one of the causes of age-related cognitive impairment observed in the aged population (Koprowska et al., 2004). In general, a great deal of evidence shows that monoaminergic transmission changes with age (Table 1).

Noradrenaline

NA and DA synthesis begins with the conversion of Tyr to 3,4-dihydroxyphenylalanine (L-DOPA), through the rate-limiting TH. Then, L-DOPA is converted to DA by DOPA decarboxylase. Finally, DA is transported into vesicles and converted to NA by dopamine β-hydroxylase. In the CNS, the NA cells are classified in groups (A1–A7) depending on their specific location. The A1 cell group

is placed at the level of the area *postrema*. The A2 cell group is distributed throughout the dorsal vagal complex. The A3 cell group is in the medullary reticular formation. The A4 cell group surrounds the fourth ventricle. The A5 cell group is in the ventrolateral pons. The A6 cell group is located at the *locus coeruleus*, dorsally in the pons, and the A7 is in cell group at the lateral part of the pons, close to the lateral lemniscus. Peripherally, this NT is used by the sympathetic ganglia (near the spinal cord) and is also release into the bloodstream by the adrenal glands (Howorth et al., 2009).In general, NA increases heart rate and blood pressure (Yamamoto et al., 2014).The main functions of NA as a peripheral hormone are to mobilize the body for action, specifically in situations of stress or danger, to induce the so-called fight-flight response. The peak of release of NA takes place during wakefulness while it is lower during sleep. In the CNS, NA is related to the maintenance of attention, memory consolidation and cerebral plasticity (Gibbs et al., 2010; Jouvet et al., 1991).

Previous studies (Table 1) have suggested that NA does not decline during aging in areas such as striatum (Ponzio et al., 1982; Tanila et al., 1994), HPC (Luine et al., 1990; Nakamura & Ohno, 1995; Ponzio et al., 1982; Stemmelin et al., 2000), PFC (Lee et al., 1994) and hypothalamus (HT) (Carfagna et al., 1985; Stemmelin et al., 2000; Tanila et al., 1994). However, alternative authors have found different conclusions. Results from Esteban et al., (2010) and Koprowska et al., (2004) found a significant decline in NA in the striatum and in the HPC, while other studies demonstrated elevated levels in the aging group (Tanila et al., 1994).

Dopamine

DA is an organic chemical of the catecholamines and phenethylamine, an amine synthesized by removing a carboxyl group from a molecule of its precursor chemical L-DOPA, which is synthesized in the brain and kidneys. In the CNS, there are four main dopaminergic projections: The mesocortical pathway that connects the ventral tegmental area (VTA) with the PFC, related to executive functions. The mesolimbic pathways, which connect the VTA with the ventral striatum (nucleus accumbens and olfactory tubercle) and is involved in reward-related behavior (incentive, pleasure and positive reinforcement) and in aversion-related behavior (Ikemoto, 2010). The nigrostriatal pathway which connects the substantia nigra with the dorsal striatum, the main functions of which are related to motor activity, reward-related behavior and associative learning. The nigrostriatal pathway is also related to neurodegenerative diseases such as Parkinson's. Finally, the tuberoinfundibular pathway, linking the HT with the pituitary gland. This last projection influences the secretion of hormones, in particular prolactin, and its activity inhibits the release of this hormone.

DA plays a special function in reward and motivated behavior and also in cognitive processes. An experiment (Sahakian et al., 1985) carried out with male Sprague Dawley rats demonstrated that high levels of cortical DA and NA correlated negatively with the number of errors made in a learning task on a delayed spatial alternation in a T-maze. These findings support the suggestion that cortical catecholamines play a role in learning and memory processes in rodents.

In general, during adulthood DA levels decline by around 10% per decade (Peters, 2006). This decrease is especially prevalent in the HPC (Godefroy et al., 1987; Koprowska et al., 2004; Míguez et al., 1999), the striatum (Eppinger et al., 2011; Esteban et al., 2010; Lee et al., 1994; Moretti et al., 1987; Ponzio et al., 1982) and the PFC (Eppinger et al., 2011; Lee et al., 1994; Míguez et al., 1999) (Table 1). In contrast, the HT seem to maintain similar levels in the aged animals (Lee et al., 1994; Sirviö et al., 1994; Stemmelin et al., 2000) and in HPC in some experiments (Luine et al., 1990; Nakamura & Ohno, 1995; Rodríguez-Gómez et al., 1995; Sirviö et al., 1994; Stemmelin et al., 2000). In relation to 3,4-Dihydroxyphenylacetic acid, DOPAC, the main DA metabolite, authors suggested decreased levels in aged compared to young animals in the striatum (Carfagna et al., 1985; Esteban et al., 2010; Moretti et al., 1987; Ponzio et al., 1982), but similar levels in the HPC (Godefroy et al., 1987; Koprowska et al., 2004; Rodríguez-Gómez et al., 1995; Sirviö et al., 1994; Stemmelin et al., 2000). In general, these findings, and the ones related to NA, suggest that an age-dependent deficit in motivated behavior or cognitive processes such as learning and memory are probably related to a reduced number of NA/DA neurons or a reduction in their activity. In addition, in the PFC, the release of DA induced by external mild stressors decreases with age (Del Arco et al., 2011). This deficit may change or slow down escape behavior when faced with external threats or stressors.

Serotonin

5-HT synthesis begins with the conversion of Tryp to 5-hydroxytryptophan (5-HTP) by the enzyme TPH, the limiting step of the pathway, as it depends on the availability of tryptophan. 5-HTP is rapidly decarboxylated and converted to 5-HT by the AADC enzyme. The enzyme monoamine oxidase (MAO) degrades 5-HT producing 5-hydroxyindolacetic acid (5-HIAA), the main metabolite. 5-HT is primarily found in the gastrointestinal tract, blood platelets, and in the CNS of mammals. The 5-HT cells inside the CNS are located in different nuclei, near the midline of the brain stem (the caudal linear nucleus, median raphe nucleus, the dorsal raphe nucleus, the nucleus raphe obscurus, nucleus raphe pallidus, nucleus raphe magnus, the intermediate reticular nuclei, and the area *postrema*) (Jacobs & Azmitia, 1992). It has been shown to be implicated in learning and memory and in practically every type of behavior, such as emotional,

motor, appetitive, cognitive and the those related to autonomic functions (Godefroy et al., 1987).

During aging, a decline in 5-HT (Table 1) has been described and it has been suggested that this decrease may contribute to behavioral changes in the normal aged population (Harada et al., 2013). In aged rats, the concentration of 5-HT in the FC (Eppinger et al., 2011), in the striatum (Eppinger et al., 2011; Esteban et al., 2010; Koprowska et al., 2004; Luine et al., 1990; Moretti et al., 1987) and in the HPC (Esteban et al., 2010; Koprowska et al., 2004; Lee et al., 1994) is reduced. Other authors have found alike levels of 5-HT and their metabolites in the same learning and memory related brain regions when comparing young and aged animals (Luine et al., 1990; Ponzio et al., 1982; Stemmelin et al., 2000). Even the ratio of 5-HIAA/5-HT, which links the metabolite levels with the serotonergic transmission, and is used as an estimator of serotonergic activity by the catabolic rate of MAO, was increased in the amygdala, striatum and FC (Stemmelin et al., 2000). Surprisingly, in some studies (Godefroy et al., 1987; Lee et al., 1994; Sirviö et al., 1994) increased levels of 5-HIAA, the main metabolite of 5-HT, have been found in aged animals. In general, these findings seems to suggest a diminution of the synthesis and the accelerated metabolism of 5-HT during aging (Stemmelin et al., 2000).

Table 1: Effects of aging compared to young control subjects on dopamine (DA), 3,4-dihidroxifenialcetic acid (DOPAC), homovanillic acid (HVA), noradrenaline (NA), serotonin (5-HT) and 5-hidroxiindolacetic acid (5-HIAA) levels in striatum, hippocampus, hypothalamus and prefrontal cortex (↓decrease, ↑increase, = no variations).

Reference	Striatum	Hippocampus	Hypothalamus	Prefrontal
Ponzio et al., 1982	↓DA, ↓ DOPAC =NA, HVA	=NA, 5-HT, 5-HIAA		
Carfagna et al., 1985	↓DA, DOPAC, HVA		↓DA =DOPAC, NA	
Moretti et al., 1987	↓DA, DOPAC, 5-HT, =HVA ,5-HIAA			
Godefroy et al., 1987	↓ HVA ↑5-HIAA = DA, DOPAC, 5-HT	↓DA, HVA ↑5-HIAA = DOPAC, 5-HT		
Luine et al., 1990	↓ 5-HT = DA, DOPAC, HVA, 5-HIAA	= DA, NA, 5-HT, 5-HIAA		

Sirviö et al., 1994		↑5-HIAA = DA, NA, DOPAC, 5-HT	= DA, DOPAC, 5-HT, 5-HIAA	
Tanila et al., 1994	=NA	↑NA	=NA	
Lee et al., 1994	↓DA, =HVA	↑ 5-HIAA, ↓5-HT =DA	=DA	↓DA, =NA
Nakamura et al., 1995		=NA, DA, 5-HT		
Rodríguez-Gómez et al., 1995		=DA; DOPAC		
Míguez et al., 1999		↓ DA		↓ DA,
Stemmelin et al., 2000		=NA, DA, DOPAC, 5-HIAA, 5-HT	↓DA = DOPAC, NA, 5-HT, 5-HIAA	
Koprowska et al., 2004	↓ HVA, NA, 5-HT = DA, DOPAC, 5-HIAA	↓DA, NA = DOPAC, 5-HT, 5-HIAA		
Esteban et al., 2010	↓ HVA, 5-HIAA, 5-HT, DA, DOPAC	↓5-HIAA, 5-HT, NA		
Eppinger et al., 2011	↓ DA, 5-HT			↓ DA, 5-HT

1.1.5 Hormones regulation

Most hormones that directly or indirectly affect the brain are produced in the pituitary gland, a gland that depends in part on the HT, which in turn, regulates its secretion. The posterior zone of the pituitary gland stores hormones produced in the HT. The anterior part produces hormones that affect growth and several glands such as testes, ovaries and breasts. The pituitary and HT brain regions are connected by a capillary system called the portal system (Osamura, 1983). The importance of this system is that it transports the hypophysiotropic hormones secreted by the HT to regulate adenohypophysial secretion. Other hormones are created in peripheral organs such as the adrenal gland above the kidneys, which produces corticosteroids and androgens. However, they also exert their function on the brain under the regulatory influence of the hypothalamus-pituitary-adrenal (HPA) axis.

As we age, changes naturally occur in the way body systems are controlled. Some tissues become less sensitive to their regulatory hormone. In addition, the number of hormones

produced by the different glands may also change (Goldman et al., 2011). For example, one of the most sensitive pathways related to the availability of the energy needed for body functions is the insulin/IGF-1 signaling pathway. During aging, the pattern of insulin levels in plasma decreases. Insulin is a peptide hormone, produced in the pancreas, which is considered to be the main anabolic hormone for body changes. It regulates the metabolism of carbohydrates, fats and proteins. It has been suggested that age-related higher circulation of insulin as well as insulin resistance are important contributors to progressive cognitive impairment and neurodegenerative processes that occur during aging (Baranowska-Bik & Bik, 2017). However, enhanced levels could positively influence emotion and higher cognitive processes including attention, learning, executive function and memory (Akintola & van Heemst, 2015). Furthermore, IGF-1 is a protein that participates in the regulation of several cellular processes in different tissues. It is produced in two places: 95% is from the liver, from endocrine action and dependent on growth hormone (GH) levels, and the remaining 5%, autocrine/paracrine action synthesized in peripheral tissue (Yamamoto & Murphy, 1995). In addition, the serum levels of IGF-1 are affected by other factors, such exercise, food intake and aging (Svensson et al., 2006). Although studies of its effects on the nervous tissue (Sonntag et al., 2005) do not allow to differentiate their origin, its presence is associated with neurotrophic effects and to the recovery of cognitive alterations related to age-dependent memory decline.

In addition, the trophic hormones, which have effects on strength and body composition, are at higher levels during puberty. These include sex steroids (androgens, estrogens and progestogens) and GH. The levels of these hormones decline progressively during aging, accompanied by a loss of muscle mass and aerobic capacity and an increase in abdominal fat in humans (Chertman et al., 2000). However, these hormones not only effect peripherial areas, but also memory and cognitive function, that progressively deteriorate with age. In this sense, an association between age-related deterioration of deep sleep (slow-wave), and a decrease in nighttime GH secretion has been found (Ho et al., 1987). This correlation may be linked to clinical problems such as sleep disorders that become significant during aging.

Other hormone levels may also be affected during aging. As we will examine in the next section, (2.2) aging has also been linked to elevated levels of cellular processes such as oxidative stress and inflammation. This increase also leads to insulin and leptin resistance and a dysregulation of energy homeostasis (Filippi & Lam, 2014). Leptin is a hormone made by adipose cells that helps to regulate energy balance by inhibiting hunger (Brennan & Mantzoros, 2006). In addition, this hormone is counteracted by the activity of ghrelin, a hormone which induces hunger. The action of both hormones are mainly focused in the HT, a crucial region for signal integration

from the peripheral pathways which plays an important role in appetite regulation (Suzuki et al., 2010). In addition, insulin and leptin are related to the metabolism of carbohydrates, proteins and fats by promoting the absorption of glucose, a process that is impaired during aging. In general, aging affects the entire body, and deregulates many of the functions that were previously controlled. For this reason, the whole process of aging brings about changes at a cellular level that end up leading to variations in behavior, generating an age-related performance with implications not only in the personality, but also in the learning and memory processes.

1.2 Neurocognitive changes

Age-associated cognitive decline or normal cognitive aging include several changes in cognition which are not uniform across mental domains or across older individuals. Age-related cognitive changes are connected to the interaction between diverse genetic, neurobiological and environmental factors. Some cognitive abilities, such as knowledge of vocabulary, are resistant to brain aging and may even improve with age. However, other abilities, such as conceptual reasoning, declarative memory and processing speed tend to gradually decline over time (Harada et al., 2013). Processing speed, for instance, begins to decay in the third decade and continues throughout the lifespan of humans (Carlson et al., 1995).

Throughout aging, the mechanisms related to learning and memory are also affected. For example, learning is a process based on the acquisition of knowledge and information about a specific stimulus in a particular environment. It involves variations in the CNS that lead to lasting changes in behavior over time. During learning, the brain rapidly forms an initial neural representation of the new experience. This is then consolidated in an organization that is optimized with the retrieval when an associated stimulus to the initial experience is cued, which forms memory (Preston & Eichenbaum, 2013). In general, cognitive processes that require HPC (for example, declarative memory) (Morrison & Baxter, 2012), and the FL integrity (for example, working memory) are most vulnerable to aging (Bizon et al., 2012; Hernandez-Ramos & Cansino, 2011). In addition, the aging process usually entails neural plasticity and synaptic transmission deficits. Synaptic plasticity is the ability of the synapses to strengthen or weaken over time, in response to changes in their activity, which aids retrieval memory processes. Therefore, the higher the level of plasticity in the CNS, the higher the chances of learning and retention taking place. In this context, the PFC and the HPC are two brain areas specifically involved in functions such as learning and memory. All these cognitive age-related alterations can be explained by

underlying neurobiological mechanisms such as changes in neural plasticity, LTP deficit, difficulties in LTD induction, or synaptic transmission deficits (Mattson & Magnus, 2006).

1.2.1. Hippocampal-dependent memory

The HPC is located deep within the medial temporal lobe of the brain, in both hemispheres, ventral to the corpus callosum and near the amygdala. It is part of the so-called hippocampal formation which consists of four brain regions: the HPC, which includes three sub-regions (CA1, CA2 and CA3), the DG, the subicular complex, with three sub-regions: subiculum (SUB), the presubiculum and the parasubiculum, and the entorhinal cortex (EC) (Figure 2b). The trisynaptic circuit is the main circuit that connects the hippocampal cells inside the hippocampal formation. It is made up of the perforating pathway that connects the EC to the DG, the mossy fibers or path that connects the DG with CA3 (Amaral & Witter, 1989) and the Schaffer collateral path that connect CA3 with the dendrites of CA1 (Figure 2a). This last path is the most relevant to the HPC, and has been the subject of many studies because it relevance for the induction and maintenance of the LTP. Finally, there are also other connections, such as those established between CA1 neurons and the EC and the paths within the SUB circuit (Cherubini & Miles, 2015).

The HPC is essential for the rapid formation and consolidation of new information into permanent memories of explicit (episodic and semantic) and context dependent spatial learning (El-Falougy & Benuska, 2006). This form of long-term memory is remembered consciously in humans and requires the involvement of the HPC (Bizon et al., 2012), which works as a sequence repeater, replaying experiences and memories when they are evoked (Preston & Eichenbaum, 2013). During aging, the physical structure of the HPC shrinks and, as mentioned before, the levels of synaptic proteins, NT and receptors related to learning and memory decrease. In fact, experimental studies on cognitive aging in rodents (Bettio et al., 2017) and humans (Nyberg, 2017) reveal deterioration of both hippocampal anatomy and physiology. Therefore, the tasks most commonly used to assess cognitive deficit in rodents are hippocampal-dependent, such as spatial learning and recognition memory. Spatial learning models consist of navigation mazes in which the encoded information is based on spatial orientation and the surrounding environment. The most widely used spatial learning and memory task is the MWM followed by different radial mazes (Adams et al., 2008; Bettio et al., 2017; Vorhees & Williams, 2006). Aged rats have shown to have an impaired performance in these kind of tasks (Portero-Tresserra et al., 2018). On the other hand, recognition memory is related to the ability to remember a previously presented item. Even though this kind of memory is less common in laboratory experiments, some tasks are focus specifically on this sort of memory recall. The task most

widely used are object recognition with a delay before the new object is presented (Ramsaran et al., 2016). Both types of cognitive capacities are critically impaired in normal aging (Warburton & Brown, 2010) and their performance depends on the integrity of the hippocampal neural function, since alteration in long-term memory recall of object-location and spatial memory tasks in aged rats is associated with defective LTP and enhanced LTD in this brain area (Arias-Cavieres et al., 2017).

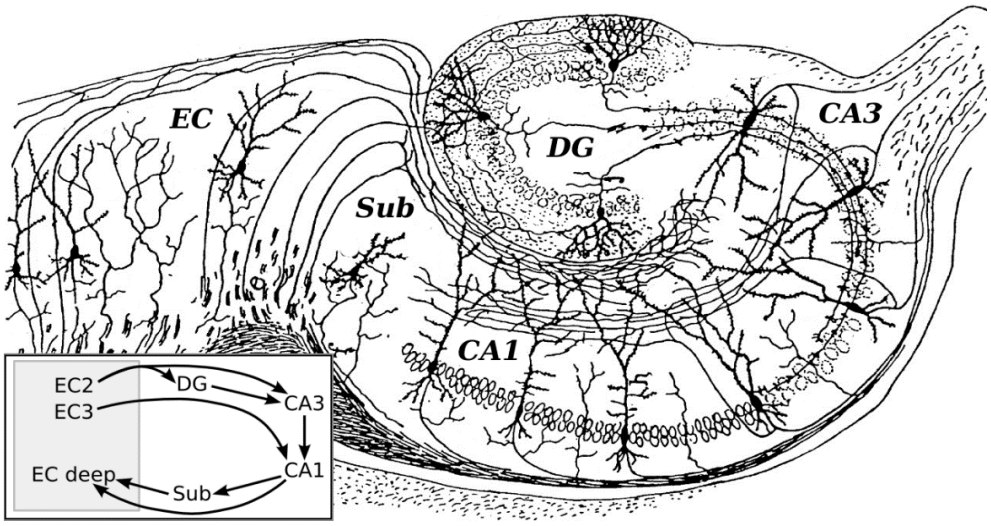

Figure 2: a) Basic circuit of the hippocampus. b) Graphic representation of the hippocampal structure DG: dentate gyrus. Sub: subiculum. EC: entorhinal cortex. (Data source: Santiago Ramón y Cajal (1911), modified).

1.2.2 Frontal lobe-dependent memory

The FL is located at the most anterior part of the brain and is the largest of the four major lobes of the cerebral cortex in the mammalian brain, taking up 41% of the total neocortical volume. It is located at the forepart of each cerebral hemisphere (in front of the parietal lobe and the temporal lobe) and separated from the parietal lobe by the central sulcus and from the temporal lobe by the lateral sulcus or Sylvian fissure. The FL can be split into the lateral, polar, orbital/ventral and medial parts and it is divided into several areas based on their function. One of the most important areas related to memory formation is the FL. It receives higher order sensory information and responds to internal and external stimuli with adaptive and flexible behavior (Catani et al., 2012). Ontogenetically this area is the last zone to develop and it is also the last to be myelinated during adulthood. The FC also manages active representations of behavioral strategies stored in long-term memory. These memories are often associated with emotions derived from the limbic system (Collins & Koechlin, 2012). The principal functions related to the FC are those related to movement control and executive function. In particular, the ability to adapt to changes, flexibility and decision-making (Collins & Koechlin, 2012). In

addition, there is also evidence that the FC contributes to memory formation through strategic control over retrieval processes within other brain areas (Postle, 2006).

The PFC is one of the sub regions of the FC located in front of the precentral area, which is related to cognition processes such as memory and executive function. The citoarquitecture of the PFC in primates differs from that of rodents; primates have 6 horizontal layers while rodents only have 4. In the cortex, pyramidal glutamatergic neurons represent about 80% of the total pool, while the remaining 20% are GABAergic inhibitory interneurons that provide local control (Buzsáki et al., 2007). The PFC is connected with a multitude of cortical and subcortical structures, which correlates with its functional involvement in various cognitive processes, thus forming an associative cortex that integrates multimodal information like cognitive, sensory, motor, emotional and autonomic inputs (Gabbott et al., 2005). In the early 90s, (Doyère et al., 1993) found for the first time that LTP occurred in the fascicle that connects the PFC to the HPC. This phenomenon of synaptic plasticity might explain the involvement of the PFC in learning and memory consolidation (DeVito et al., 2010). For this reason, injuries to this cortical area are used to produce deficits in cognitive capacity and flexibility in laboratory animals (Taylor et al., 2003). Specifically, the PFC is involved in working memory, a type of short-term memory, which refers to the temporary retention of current information about the environment in order to manipulate it and combine it with past experiences. Moreover, the PFC is also related to the cognitive flexibility, the mental ability to switch between thinking in one concept, and to think in multiple concepts simultaneously, which seem to be impaired during aging as the working memory too (DeVito et al., 2010). The PFC shows changes with aging; at a structural level, there is evidence of atrophy and loss of both grey and white matter. However, the decline in the volume of white matter is disproportionately greater, which seems to be related to changes in axonal structure, resulting in slower neurotransmission. In addition, the grey matter decline seems to be related to the reduction in the number of cells (Salat et al., 1999) or neuronal shrinkage (Kensinger, 2010). There is also a reduction in hemispheric asymmetry that could be linked to the decline in episodic memory detected in older adults (Rossi, 2004). All these structural changes are related to the alteration in the cognitive functions.

2. Caloric restriction and aging

A normal calorie consumption is essential for regulating physiological processes and supporting life, but an excess or a deficit in food ingestion may increase morbidity and mortality (Morgan et al., 2017). It is widely accepted that the rate of aging can be affected by the amount of food consuming during life and there are several studies with different organisms, from yeast to mammals, that demonstrate the beneficial effects of CR (Arslan-Ergul et al., 2013; Balasubramanian et al., 2017; Mirzaei et al., 2014). Throughout history, from the ancient Greeks and Romans to modern times, different societies have been interested in the benefits of food intake limitation (Dehmelt, 2004). Nowadays, different experiments with aged populations have been performed (Witte et al., 2009), but no detailed reports about the specific effects of a CR diet and longevity has been provided. This is mainly due to different factors such as difficulties to reach an adequate sample number, multiple different cultural related component of the diet or the effort to maintain a social accepted diet. However, it seems well-defined that a CR diet in humans might improve health span (Anderson & Weindruch, 2012).

Around 1935, a research team (Mccay et al., 1935) used a diet with CR as a means of extending the life expectancy of laboratory animals to determine the underlying mechanisms of the aging process. Results confirmed that CR was able to increase longevity in rats. Nowadays, CR is used in laboratory animals as a way of delaying the onset of the aging process and to prevent age-related disorders (Chung et al., 2013). In animal models, CR is also the only non-genetic intervention that has been proven to enhance both health and lifespan (Arslan-Ergul et al., 2013). Even the Institutional Animal Care and Use Committees (IACUCs) from the United States encourage to use CR in their *Guidelines for Diet Control in Laboratory Animals.* This guide empathizes that, although *Ad Libitum* feeding is the normal practice in the vivarium, it is not the common diet for most animals in the wild. The free-food access diet can develop to sedentary lifestyle, glucose intolerance and obese animals. The practice of CR is accepted for long-term housing of rodents and rabbits, because it favors a better living and aging.

A CR diet usually involves a reduction of between 20% and 40% of the total calorie intake compared with that of the *Ad Libitum* control group. This reduction in food intake may be maintained for long periods or applied intermittently during a specific period, but it should not be confused with short or long-term starvation. If a CR diet (in form of starvation) is applied to rats for less than 3 weeks, it induces acute stress, and this might overshadow the general positive benefits of the diet (Arslan-Ergul et al., 2013). But, if the CR diet is applied regularly for a longer period of time, the positive effects of the intervention appear and become evident. Moreover,

differences between the type of diet and the organism used, may affect the results of experiments with CR on health and longevity in animals. Due to these factors, some controversial results have been obtained (Cardoso et al., 2016). In addition, in order to obtain the beneficial effects of CR on health, it is crucial to consider the time when animals started on the CR diet, which must also be consistent with the organism and the objectives of the study. Each specific age phase has a precise nutritional need and susceptibilities. A recent study (Cardoso et al., 2016) in rats demonstrated that a 50% CR diet started at 4 weeks-old, during the animals development, affected negatively the spatial learning and memory of the animals. The rodents performed significantly worse in the Open Field (OF), the Elevated plus maze (EPM) and the MWM tasks; in addition, it disturbed hippocampal neurogenesis. In contrast, when the animals had finished their development, at the initial adult phase, they are more resistant to environmental variations, nutritional deprivations and external aggressions (Morgane et al., 1978). Therefore, if the CR is presented when the animals are fully developed and lasts for the duration of the aging process, the beneficial effects of CR become evident. In particular, animals show better performance in learning and memory tasks as well as the maintenance of stable levels of synaptic proteins in the HPC during aging (Bettio et al., 2017).

2.1 Caloric restriction implementations in human's history

The investigation of CR in humans is controversial and difficult due to moral issues (Arslan-Ergul et al., 2013). Healthy people would not agree to participate in a CR investigation unless they were ensured that the diet would slow down the aging process, as seen in rodents. In addition, the reduction of food consumption in humans is difficult due to the constant bombardment of food-related input in 21st century society. However, different, involuntary episodes of CR in human history could be considered. For example, during World Wars I and II, some countries, for example Denmark, forced the population to reduce their intake for 2 years. The results of this forced CR experiment conducted to a 34% reduction in death rates in the population between 25 and 65 years old (Hindhede, 1920). Furthermore, even nowadays, the religious fast from sunrise to sunset that Muslims practice during the month of Ramadan provides a good opportunity to study the effects of IF and CR. The outcomes of fasting indicate that during this month the plasma levels of 5-HT and BDNF significantly increase when compared to basal levels (Bastani et al., 2017). Moreover, a study (Aksungar et al., 2017) of 23 non-diabetic female subjects subjected to a 2-year CR diet, within which they had to perform the religious IF for 1 month demonstrated that this dietary intervention might enhance health and cellular resistance to disease by changing eating and sleeping patterns in the population. Other religious variations in nutrition are performed by the Greek Orthodox population, who eat a restricted vegetarian

diet for 180 to 200 days per year. This food intervention was investigated and the results indicated that the total cholesterol, body mass index (BMI) and Low density lipoprotein, was lower when compared to the basal levels registered prior to the fasting period (Papadaki et al., 2008). Furthermore, the case of Okinawa is also well known. Okinawa is a Japanese Island where 50 in every 100000 people are 100 years or older. This percentage is approximately four to five times higher than that of the general population in Japan (Most et al., 2017). Currently, both the average and maximum lifespan is higher on Okinawa (average: 83.8 years, maximum: 104.9 years) compared to the population of continental Japan (average: 82.3 years, maximum: 101.3 years) (accurate as of 1995) (Willcox et al., 2007). In 1972, a poll conducted by the Japanese National Nutrition survey suggested that the population of Okinawa consumed only 83% of the average Japanese caloric intake (Kagawa, 1978), which is 40% less than the average of caloric intake of adults in the United States (Most et al., 2017). In general, the diet of the Okinawans consists of different green leafy vegetables, soy, sweet potatoes and a small amount of animal protein (9% of total calories intake) such as fish (Redman et al., 2008; Willcox et al., 2007). Remarkably, this diet has the optimum content of dietary protein and carbohydrates, which is almost identical to the one used in laboratory animals nowadays (Le Couteur et al., 2016). In conclusion, all these findings suggested that a lower calorie intake and a negative energy balance during the first stages of life result in a low BMI, decreased risk of age-dependent diseases and an extended lifespan (Roth & Polotsky, 2012).

2.2 Mechanisms of action of caloric restriction

Although the exact mechanisms through which CR promotes lifespan are still not fully understood, different studies have demonstrated the beneficial effects of this dietary intervention on the aged brain (Table 2).

2.2.1 Metabolic and hormonal changes

Part of the beneficial effect of CR on health is due to the regulation of insulin/IGF-1 levels, which drop moderately following the dietary intervention. In addition, CR decreases insulin resistance and conserves blood glucose within healthy baseline values in aged subjects (Speakman & Mitchell, 2011). In fact, a study performed in humans (Fontana et al., 2010) showed that a CR diet for a long period of time (6.9 ± 5.5 years) increased levels of adiponectin, a hormone involved in regulating glucose levels as well as fatty acids breakdown and improved insulin sensitivity. However, around 40% of the participants displayed exaggerated hyperglycemic response to glucose load and an impaired glucose tolerance, which was associated with lower levels of IGF-1 and triiodothyronine, a hormone of the thyroid gland. Moreover, a two-year CR

diet study (Aksungar et al., 2017) in obese humans indicated that over time, all subjects lost weight and the levels of blood glucose and insulin increased. Conversely, the levels of IGF-1 decreased progressively until the end of the follow-up. In humans, high plasma levels of IGF-1 have shown to be a risk factor for cancer, but can also protect against age related illness like diabetes mellitus (type 2), cognitive decline and osteoporosis (Barzilai & Bartke, 2009). Similarly, studies performed in non-human primates (Kemnitz, 2011) showed increased insulin sensitivity and glucose tolerance in old animals.

In rodents, different and controversial results about the regulation of GH and IGF-1 were found. Some authors demonstrated that long-term CR increases GH and IGF-1 levels (Sonntag et al., 2005), although other found out that CR decreases serum IGF-1 concentration by 40% compared to *Ad libitum* animals (Fontana et al., 2008). In addition, previous investigations (Novelli et al., 2004) into insulin hormone levels showed that a lifelong 40% CR counteracts the decrease in adipocyte insulin-stimulated lipogenesis in 24 month-old Sprague rats, which might contribute to an imbalance in fat distribution in some tissues. Furthermore, two months of moderate 16% CR has been shown to reduce serum leptin in young and aged rats (Niemann et al., 2008). In general, CR enhances the levels of hormones such as glucocorticoids that seem to be affected during aging, and to extend lifespan. The presented results demonstrated that an adaptation of the CR studies performed in rodents might have the same beneficial effects in humans as well (Fontana et al., 2010).

Regarding inflammatory processes, previous results (Solana et al., 2006), have shown that CR decreases the expression of genes associated with inflammatory responses like tumor necrosis factor (TNF-α) and interferon gamma (IFN-ɣ), cytokines involved in the regulation of immune cells, which normally increase during aging due to oxidative stress, a topic that will be explored further in subsequent sections.

Table 2: Summary of the different effects of CR diets on health in several organisms (modified from Van Cauwenberghe et al., 2016).

Mechanism	Observed beneficial effects	Organism
Metabolic effects	Lowered body temperature	Rodents, primates, humans
	Reduced metabolic rate	Rodents, primates, humans
	Reduced adipose tissue	Yeast, rodents, primates, humans
	Improved glucose regulation	C.elegans, rodents, humans
Immunological and hormonal changes	Increased insulin sensitivity	Rodents, primates, humans
	Reduced inflammation	Yeast, rodents, mammals
Epigenetic modifications, and Neuroprotection	DNA methylation changes	Rodents, mammals, humans
	Decreased ROS	Yeast, rodents, primates
	Hormesis: production anti-oxidants, DNA repair enzymes and anti-apoptotic proteins	Rodents, primates
	Increased levels of neurotrophic factors (BDNF)	Rodents, primates
Autophagy, apoptosis and cell survival	Downregulation of p53 and SIRT1	Yeast, C.elegans, Drosophila, rodents, mammals, humans
	Inhibition of mTOR pathway	C.elegans, Drosophila, mammals
Oxidative stress	Reduced oxidative damage	Yeast, rodents, primates

2.2.2 Epigenetic, genetic changes and neurogenesis

In general, tissue of SNC shows a high level of plasticity only comparable to that of an embryo (Urdinguio et al., 2009). This makes it very sensitive to manipulation, especially those involving dietary interventions (Wood et al., 2015). One of the main effects of CR in aged animals is that it promotes the expression of multiple genes, 25% of which are involved in synaptic plasticity (Park & Prolla, 2005), which might affect memory and learning processes. Additionally, CR has been shown to increase the synaptic activity of neural circuits and the levels of phosphorylated cyclic adenosine monophosphate response element-binding protein (pCREB), a cellular transcription factor which binds to certain Deoxyribonucleic acid (DNA) sequences.

During aging, cells accumulate DNA damage due to oxidation and to the progressive deterioration of their structure due to mutations and epigenetic alterations such as methylation and histone modifications. This is the main reason why at advanced ages, loss of telomere integrity is observed, as well as a global reduction in DNA methylation, which produces genome instability (Wątroba et al., 2017). Several studies (Li et al., 2011; Moreno & Mobbs, 2017) seem to indicate that diet can alter DNA methylation patterns, affecting genes linked to neurodegeneration through the modification of the activity of methyltransferases (DNMTs), which are involved in the control of DNA transcription. CR, as a dietary intervention, exerts a neuroprotector effect during aging because it improves the state of oxidation of brain cells while it modifies the regulation of genes. In addition, CR recovers the levels of methylation by the action of DNA methyltransferase 3a (DNMTA3a) which catalyzes DNA methylation. This protein is essential for memory formation and for the underlying changes in neuronal and synaptic plasticity. CR has also been shown to attenuate the age-related decrease in DNMTA3a in the HPC of mice (Chouliaras et al., 2011) which might contribute to improved learning and memory capacities during age.

In addition, CR increases the expression of neurotrophins like BDNF in CA1 (Newton et al., 2005). BDNF is made in the endoplasmic reticulum and secreted in vesicles. This neurotrophic factor is especially active in learning and memory related brain areas such as HPC and FC. The main functions of BDNF are related to supporting the survival of existing neurons and contributing to the general stability of the total number of synapses per neuron in the HPC by supporting synapse function. It is well established that this process is downregulated during aging (Newton et al., 2008) and reversed by the dietary intervention. CR also seems to modify genes related to synaptic plasticity and mitochondrial function in the PFC (Del Arco et al., 2011). These genes are sensitive to external changes or processes like aging and undergo downregulation at the same time as the immune response is increased in adult and elderly humans (Lu et al., 2004). However, CR is able to change the activity of these genes and to slow down the age-related loss of synaptic plasticity. In fact, In vitro CR intervention ameliorated the pathways related to neurodegeneration in aged mice (García-Matas et al., 2015). It is also interesting to note that one of the molecular bases underlying the conservation of cognitive capacity induced by CR during aging seems to be the maintenance of the transcriptional coactivator peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1α). This protein activates transcription factors involved in the regulation of mitochondrial biogenesis and antioxidant response (Fontán-Lozano et al., 2008).

Furthermore, CR contributes to the maintenance of neurogenesis in the DG of the HPC at advanced ages (López-Lluch & Navas, 2016). The application of CR for short or long periods has been shown to preserve stem cell population (Apple et al., 2017). This process occurs throughout life in different zones of the brain such as the ventricular-subventricular zone (V-SVZ) of SGZ of the DG in the HPC. However, during aging, neurogenesis declines and slows down. In fact, the V-SVZ was found to be thinner in aged mice compared to younger control animals (Luo et al., 2006). As described above (Section 1.1.1), reduced neurogenesis seems to contribute to age-related cognitive impairment and a decrease in brain plasticity (Apple et al., 2017). Previous investigations (Phillips, 2017) demonstrated that diet and physical activity modulate neurotrophic signaling, neurogenesis and stress response in the brain areas related to cognition during aging. Thus, a long term CR diet was able to maintain the proliferation of progenitor cells in the HPC in aged female mice (Park et al., 2013). Furthermore, similar dietary restriction interventions seem to enhance neurotrophin expression, BDNF and neurotrophin-3, and neurogenesis in the DG of the HPC, by increasing the cell survival of adult, 8-week-old, mice (Lee et al., 2002). However, other studies found controversial results. For example, a study (Bondolfi et al., 2004) with elderly C57BL/6 mice revealed a substantial reduction in neurogenesis in the DG, and long-term CR did not counteract this age-related decline, however, it did promote survival of newly generated hilar glial cells of the DG.

2.2.3 Apoptosis, autophagy, cell survival and oxidative stress

To ensure that cells function properly, the brain requires the elimination of structures and molecules damaged via apoptosis, which is a process of programmed cell death that occurs in multicellular organisms. This procedure is important for the regulation of normal cell turnover, proper development and functioning of the immune system, embryonic development and chemical-induced cell death (Elmore, 2007). Inadequate apoptosis, whether too high or too low, is a significant factor in many human alterations including neurodegenerative diseases or ischemic damage. In addition, an alternative process for the elimination of damaged structures is autophagy, which is performed through the ubiquitination of molecules that must be eliminated or through the lysosomal autophagic system, which removes cellular organelles and molecular aggregates (Das et al., 2017). This process is regulated by nutrient availably and monitored by nutrient sensors and cascades such as 5' AMP-activated protein kinase (AMPK) and target of rapamycin in mammals (mTOR) that are usually downregulated during aging. A particular form of autophagy is mitophagy, a selective process of mitochondria degradation by autophagy (Morgan et al., 2017). This procedure is sensitive to food intake. The decrease in

autophagy and mitophagy is a characteristic of the body's response to hypercaloric diets and involves the acceleration of aging processes (Pani, 2015).

Another important factor is that CR exerts its effect on the age-related decline of mitochondrial turnover caused by mitophagy. The dietary restriction prevents the inhibition of this process by maintaining only the healthy mitochondria in the cell and eliminating the damaged ones (López-Lluch & Navas, 2016). This action is based on the effects of CR on the family of oxidized Nicotinamide adenine dinucleotide (NAD^+) dependent protein deacetylases, the sirtuins, found in all organisms. Sirtuins affect the activity of many enzymes by removing acetyl residues. In mammals, CR induces the activity of sirtuins, which seem to play a central role in aging and longevity. In addition, sirtuins are important for the regulation of fundamental biological responses to a variety of environmental and nutritional indicators (Imai & Guarente, 2010). These proteins act as a nutrient and metabolic sensor by detecting fluctuations in NAD^+/NADH (reduced form of NAD^+) ratio. When nutrients decrease, NAD^+ accumulates and sirtuins are activated leading to antioxidant protection of the cell (Guarente, 2013). The overexpression of NAD-dependent deacetylase sirtuin-1 (SIRT1) in the brain extends lifespan and delays aging in mice (Satoh et al., 2013). It can also prevent axonal degeneration after an injury or neurodegeneration, and acts against β-amiloid peptide accumulation (Bishop et al., 2010). SIRT1 plays a central role in inducing stress tolerance, mitochondrial biogenesis and fat metabolism. These actions support the proposal that sirtuins are conserved mediators of longevity (López-Lluch & Navas, 2016).

Disturbances in the normal redox state of cells, a chemical reaction in which the oxidation states of atoms are changed, can lead to toxic effects in cell production of free radicals or peroxides that damage the components of the cell (Hayyan et al., 2016). During normal aging, and in times of environmental stress, generation of reactive oxygen species (ROS) increases, modifying cell signaling and homeostasis (Devasagayam et al., 2004) which leads to oxidative stress. Oxidative stress reflects an imbalance between the manifestation of ROS and the underlying biological system in charge of detoxifying the reactive intermediates or repairing the resulting damage. *Ad Libitum* aged mice, with higher body weight and abdominal fat positively demonstrated higher levels of brain oxidative damage (García-Mesa et al., 2015). In fact, CR has been shown to lower the levels of ROS in mammals and similar findings have been discovered in rodents. This can be explained, in part, by the fact that CR provides a higher level of protection against increases in oxidative stress and subsequent cell damage via a decrease in the age-dependent inhibition of mitophagy (López-Lluch & Navas, 2016). CR lowers mitochondrial membrane potential and as a

consequence the production of ROS, which modifies the saturation or unsaturation index of the mitochondrial membranes, thus preventing oxidative damage (Chen et al., 2012).

If sufficient energy is available in a cell, anabolic processes are activated. Such processes trigger mTOR, which may be related to cell duplication (Huang & Fingar, 2014). The mTOR signalling controls growth, cell survival, autophagy and metabolism in the cell. The activity of mTOR is regulated by insulin, rapamycin, oxidative stress and growth factors such as IGF-1 (Yang et al., 2014). CR seems to decrease or inhibit mTOR signaling, resulting in an increase in lifespan, and healthier state of the different tissues, including the nervous system (Bjedov & Partridge, 2011). However, it is clear that other pathways contribute to the overall health benefits observed in CR animals. These are likely to include insulin/IGF signaling and AMPK activation (Goldberg et al., 2015). AMPK, a detector of low energy, has been described as a molecular transducer of starvation signals, acting as a nutrient sensor with the ability to regulate whole-body metabolism (Canto & Auwerx, 2011). This protein is necessary to extend lifespan and attain longevity in all species. When AMPK is stimulated, it activates a catabolic pathway to re-establish adenosine triphosphate (ATP) levels, in the short term by endorsing glycolysis and fatty acid oxidation, and in the long term by using the mitochondrial substrates as an energy source (Cantó & Auwerx, 2010). Furthermore, AMPK can inhibit the mTOR cascade through the phosphorylation and activation of an inhibitory tuberous sclerosis complex (TSC), which also regulates autophagy through the phosphorylation of UNC-51-like kinase 1 (ULK1). CR can stimulate AMPK activity, which coordinates the signaling of many age-related transcription factors, whereas an excess caloric diet seems to impair this pathway (Salminen & Kaarniranta, 2012).

2.2.4 Monoaminergic transmission and synaptic plasticity related brain proteins

It has been observed that CR may reduce the effects of aging on glutamatergic transmission in the HPC, cortex and cerebellum (Monti et al., 2004) (Table 4), and attenuate the aging-dependent decline of monoamine levels (Del Arco et al., 2011; Maswood et al., 2004; Michalsen, 2010) (Table 3). Interestingly, CR is associated with greater synaptic and electrical activity in neural circuits, steadying levels of Glu receptors and proteins required for excitatory transmission. This effect may provide optimal neural conditions for hippocampal-dependent learning and memory processes (Adams et al., 2008).

Monoaminergic transmission

In relation to the monoaminergic NTs, CR application has been shown to be an effective way to combat age-related loss of monoamines (Table 3). DA related NTs and proteins were especially enhanced in the striatum (Maswood et al., 2004) and in the HPC (Del Arco et al., 2011), but not

in the PFC where *Ad Libitum* animals showed similar levels than the CR group (Del Arco et al, 2011). These results suggest that CR does not modify the function of the PFC in relation to DA levels as a result of the normal process of aging, although it does seem to have effects in the striatum and HPC, both of which are brain areas related to cognitive processes such as learning and memory (Del Arco et al., 2011). On the other hand, when it comes to serotoninergic related NTs and proteins, different results have been described. In general, in CR aged animals, enhanced levels of 5-HT in the PFC were detected compared to the *Ad Libitum* group (Michalsen., 2010). In relation to the HPC, decreased levels of 5-HIAA and similar 5-HT levels (Jahng et al., 2007) were found. Finally, levels of 5-HIAA in the HT were similar, but, elevated levels of 5-HT in the same area were discovered (Jahng et al., 2007). In general, these findings indicate that both NTs systems are part of the underlying mechanisms of the influence of dietary interventions such as CR.

Table 3: Effects of caloric restriction during aging compared to Ad Libitum animals on dopamine (DA), 3,4-dihidroxifenialcetic acid (DOPAC), homovanilic acid (HVA), noradrenaline (NA), serotonin (5-HT) and 5-hidroxiindolacetic acid (5-HIAA) levels in striatum, hippocampus, hypothalamus and prefrontal cortex (↓decrease, ↑increases, = no variations).

Reference	Striatum	Hippocampus	Hypothalamus	Prefrontal
Del Arco et al., 2011		↑DA		=DA
Jahng et al., 2007		↓5-HIAA, =5-HT	=5-HIAA, ↑5-HT	
Maswood et al., 2004	↑DA, DOPAC, HVA			
Michalsen, 2010				↑5-HT

Glutamatergic receptors and proteins involved in synaptic plasticity

Age-related deficits and their impact on cognitive tasks are mainly related with hippocampal and prefrontal functions and with the loss of synaptic plasticity. Dietary intervention such as CR can maintain a stable number of Glu receptors in the HPC of CR aged rats in comparison to their younger counterparts. In contrast, older *Ad Libitum* fed rats tend to show a general decrease in the levels of these receptors compared to young control animals (Table 4). For example, there is a dramatic decrease in the expression of the NMDA receptor subunit NMDAR1 in *Ad Libitum* aged rats when compared to CR fed animals (Eckles-Smith et al., 2000). In addition, not only did the CR old rats have the same levels of NMDAR1 subunits compared to the young ones, but also

the levels of NMDAR2A and NMDAR2B subunits were stable over time. Moreover, CR eliminated the age-related decline of NMDA and AMPA receptors in aged Fischer 344xBrown Norway rats of 10, 18 and 29 month old by inducing stable levels over time (Shi et al., 2007).

In relation to SYP, previous studies (Schmitt et al., 2009) with SYP knockout mice proved that these animals showed impairments in object novelty recognition and spatial learning, demonstrating that SYP is involved in learning and memory processes. In addition, SYP expression enhancement may not only restore memory, but also enhance spatial memory, due to improvements in synaptic plasticity (Hajjar et al., 2013). In addition, SYP deficits in HPC during aging are related to a worse performance in the MWM task (Portero-Tresserra et al., 2018). In fact, age-related neural protein loss could be due to a drop in the number of synapses and/or to an altered synaptic configuration (Adams et al., 2008). As previously described, CR can maintain stable levels of learning and memory related brain proteins. Earlier results (Smith et al., 2007) indicated that 24 month old rats had less expression of SYP, but CR seemed to attenuate this loss in the HPC (Adams et al., 2008; Singh et al., 2015) (Table 4).

Table 4: Effects of caloric restriction diet on receptors levels (NMDAR1, NMDAR2A, NMDAR2B, AMPA1 and AMPA2) and synaptic plasticity protein (SYP) in HPC, the comparison are referenced to young control groups of animals for each experiment. Old CR: Aged caloric restricted group of animals. Old AdL: Aged Ad Libitum group of animals (= equal levels in experimental group and control) (↓ significant less levels of receptor in experimental group compared to control young group.

Reference	NMDAR1	NMDAR2A	NMDAR2B	AMPA1	AMPA2	SYP
Eckless-Smith et al., 2000	Old CR = Old AdL ↓			Old CR = Old AdL =		
Monti et al., 2004	Old CR = Old AdL ↓	Old CR = Old AdL =	Old CR = Old AdL =			
Shi et al.,2007	Old CR = Old AdL ↓	Old CR = Old AdL ↓	Old CR ↓ Old AdL ↓	Old CR = Old AdL ↓	Old CR = Old AdL =	
Adam et al., 2008	Old CR = Old AdL ↓	Old CR = Old AdL ↓	Old CR = Old AdL ↓	Old CR = Old AdL ↓	Old CR = Old AdL ↓	Old CR = Old AdL ↓
Newton et al., 2008	Old CR = Old AdL ↓	Old CR = Old AdL =	Old CR ↓ Old AdL =	Old CR = Old AdL ↓	Old CR ↓ Old AdL =	

Sigh et al., 2015						Old CR = OldAdL ↓
Potero-Tresserra et al.; 2018						Old CR = OldAdL ↓

2.2.5 Negative effects of caloric restriction diet

Besides all the beneficial effects of CR, several studies have reported some detrimental effects of this dietary intervention. For instance, reductions in reproductive state have been detected (Adler & Bonduriansky, 2014), as well as poorer immune response (Ayres & Schneider, 2009). CR fed rodents are more susceptible to infection by intact pathogens than *Ad Libitum* fed animals (Kristan, 2008). Other authors (Andrade, et al., 2002) have found that the total number of GD granule cells and hippocampal CA3 neurons did not differ between animals on a 40% CR diet for 36 weeks and their *Ad Libitum* feed control rats. However, even though there were no changes in the number of cells, the number of segments of dendritic arborization of granule cells were diminished in the CR group of rats.

Other negative effects of CR include changes to subjects' emotional state. Firstly, monoamines play a primary role in depression, lower levels of these NT may be linked to this disorder (Hirschfeld, 2000). Moreover, elevated levels of plasma corticosterone can aggravate it, adding a component of anxiety-like behavior (Gregus et al., 2005). A previous investigation (Jahng et al., 2007) in rats revealed that after a five weeks of 50% restricted diet, levels of 5-HT in the HPC and HT were decreased and corticosterone levels as well as the results of specific behavioral tests demonstrated that young rats in chronic CR may develop a depressive and/or anxiety disorder. For these reasons, a good dietary intervention, with a controlled percentage of food reduction and animals housed in pairs for social interaction are important requirements to achieve the beneficial effects of CR and to avoid the aforementioned detrimental repercussions.

2.3 Cognitive effects of caloric restriction

As stated above, CR is one of the most effective dietary interventions known to date to prevent age-related cognitive decline and promote a better health span by delaying the onset of age-related diseases in different animal models (for a review see Speakman & Mitchell, 2011).

In this section, we present different studies of CR and aging and its effect on cognitive performance in rodent (rats and mice) and humans.

2.3.1 Studies in rats

In general, rats are the most commonly used experimental animals in numerous biomedical studies, as they have been recognized as the preeminent model for aging studies due to the ratio from rat to human years. During rodent adulthood, one day is equivalent to approximately 34 human days and therefore it is accepted that a 60 year old human can be represented by a 24 month-old rat (Sengupta, 2013).

Rat strain and caloric restriction procedures

The effect of a CR diet on cognition has not been investigated in much depth and the specific results are still unclear due to contradictory findings. In rats, several experiments on the effects of CR on cognition have shown differences in learning and memory performance depending on the strain of the rat (Table 5). For example, in Sprague Dawley rodents, which is the most widely used strain to analyze CR and the effects of aging, CR has been able to enhance long-term spatial memory in aged animals compared to their *Ad Libitum* controls (Algeri et al., 1991; Geng et al., 2007; Gyger et al., 1992; Pitsikas & Algeri, 1992; Pitsikas et al., 1990; Pitsikas et al., 1991). However, contradictory conclusions were found in another strain, Wistar rats (Bond et al., 1989; Singh et al., 2015; Yanai et al., 2004). Results from one experiment on this strain (Goodrick, 1984) indicated that CR might exert a positive effect on a T maze learning task, whereas in another study (Del Arco et al., 2011) CR failed to enhance memory in the same task. In addition, olfactory tasks do not seem to be the most appropriate to analyze the effects of CR. As in one experiment (Bond et al., 1989), CR did not improve the memory in this strain. Furthermore, discordant results have also been found in the strains of Fischer 344 (F344), BN and the hybrid F344xBN rats (Adams et al., 2008; Carter et al., 2009; Fitting et al., 2008; Markowska, 1999; Markowska & Savonenko, 2002; Stewart et al., 1989). Some experiments showed that CR had a positive effect on spatial memory (Carter et al., 2009; Fitting et al., 2008; Markowska & Savonenko, 2002; Stewart et al., 1989) whereas others did not (Markowska, 1999). These inconsistent results may be due, at least in part, to the different strains used, the periods and the initial age of CR application, the age of the animals or other housing variables. These differences may affect the results and change the sensitivity of the animals to the hypocaloric diet (Mitchell et al., 2016).

Another important factor to consider when analyzing different CR experiments is the procedure of intervention (IF or continued CR). In general, CR has been shown to be more effective than IF when it comes to preventing age-related memory impairment (Gyger et al., 1992). Regarding

this procedure, experiments using IF animals which were fed every other day presented controversial results, a 24% diminution of food intake since weaning demonstrated positive effects of the IF group in the T maze (Goodrick, 1984) or no effect in 8 arm radial maze (Beatty, Clouse, & Bierley, 1987). In contrast, the application of a continued, long-term CR diet seems to be more effective for improving memory in aged animals (Del Arco et al., 2011; Markowska, 1999; Stewart et al., 1989). In this case, the percentage of CR most commonly used is around 20 to 40% of the total food intake of *Ad Libitum* animals. Higher restricted diets may simulate a semi-starvation diet (Jahng et al., 2007) leading to malnutrition which in turn affects the results. In some experiments (Swindell, 2012) the percentage of food restriction is gradually increased up to a certain point. Another study (Del Arco et al., 2011) decided on a percentage of food restriction at the beginning of the intervention, and maintained that same level throughout the course of the procedure. Additional experiments (Algeri et al., 1991; Markowska, 1999; Pitsikas et al., 1990; Pitsikas et al., 1991), added vitamin supplements to the hypocaloric diets. All these variables make comparing the results obtained from the different experiments difficult.

Behavioral tasks used to analyze learning and memory in rats

An additional factor that might interfere with the results observed between different CR studies is the type of behavioral task used in order to assess specific learning and memory processes. Most of the experiments are based on tasks such as MWM and radial mazes, both of which are spatial hippocampal-dependent memory procedures (Murphy, 2013). The most widely used cognitive task is the MWM, which assesses spatial learning and memory. This task has proved to be a robust and reliable test that correlates with hippocampal synaptic plasticity and NMDA receptor function (Vorhees & Williams, 2006). It is used in laboratories across the world and is easily reproduced with a simple procedure (Wahl et al., 2017). In general, all the interventions with CR and aging using MWM test have shown that CR has a positive effect on memory in older rats (Table 5) with some exceptions (Hansalik et al., 2006; Markowska, 1999) or only a slight effect in the CR group (Fitting et al., 2008). However, the possible beneficial effects of the CR on MWM memory have been questioned. Different authors (Kishi & Sunagawa, 2012) have proposed that the improvement in the performance of the task in older CR animals could be attributed to the increase in the motor response of the animals subjected to the dietary intervention and not to the improvement of their cognitive ability. However, research supports the hypothesis that CR enhanced spatial memory in the MWM (Kishi & Sunagawa, 2012).

Other mazes, such as 8 arm radial maze, which assesses reference spatial memory when the animal only visits the arm where the food reinforcement is, and working memory when the

animal enters each arm only a single time (Tarragon etl al., 2012) has been used less frequently, and controversial results have also been found. Some experiments (Pitsikas & Algeri, 1992) demonstrated that the execution of the CR group was enhanced in this maze. In contrast, other results did not detect differences between experimental groups (Beatty et al., 1987; Bond et al., 1989; Stewart et al., 1989). In addition, explicit memory tasks such as novel object recognition tests, which utilizes rodents' natural exploratory behavior (Bevins & Besheer, 2006) or alternating delays in an aquatic T maze have also been used. However, previous experiments indicated that using these tests, CR did not enhance performance (Carter et al., 2009; Del Arco et al., 2011). Moreover, there is less evidence from other types of tasks related to the assessment of implicit memory. For instance, CR has been shown to be able to prevent age-related cognitive impairment in a passive avoidance task (Pitsikas et al., 1991), a well-established associative memory procedure which requires the subject to behave contrary to their innate tendency. Animals are conditioned to prefer dark areas and avoid the bright ones with the application of an aversive food-shock stimulus (Wahl et al., 2017). Similarly, results from the use of food reinforced tasks such as instrumental conditioned or ODT tasks has been controversial (Beatty et al., 1987; Bond et al., 1989; Goodrick 1984). Evidence suggests that when animals are chronically deprived of calories, the reward of a food stimulus becomes more salient (Cameron et al., 2008). Moreover, dietary restriction per se might represent a stressor, and since stress can influence a range of behaviors, experiments performed with food reward can be biased (Smith & Metz, 2005). In this kind of tasks (Markowska & Savonenko, 2002; Yanai et al., 2004) it is questionable whether the level of food restriction prior to the task can modify the value of the positive reinforcement from both CR and *Ad Libitum* groups of animals. Therefore, experiments achieved using food reinforced parameters have been inconclusive. Some have shown positive effects of CR (Goodrick, 1984), others have not (Beatty et al., 1987) while others have even shown negative effects of CR compared to the control group of rats (Bond et al., 1989).

Table 5: Different experiments over time that studied the effects of CR on learning and memory of aged rats.

Author	Strain	Age (months)	Intervention	Task	Results
Goodrick, 1984	Wistar	6 m 22 m 35 m	*Ad Libitum* Intermittent fasting	T maze	Positive effects
Beatty et al., 1987	Sprague-Dawley	3 m 21 m	*Ad Libitum* Intermittent fasting	8 arm radial maze	No effects

Bond et al., 1989	Wistar	12 m	*Ad Libitum* Caloric restriction	8 arm radial maze Olfactory memory task	No effects
Stewart et al., 1989	Fischer 344	8 m 16 m 24 m 30 m	*Ad Libitum* Caloric restriction	8 arm radial maze MWM	Positive effects in MWM
Pitsikas et al., 1990	Sprague-Dawley derived CD-COBS	4 m 12 m 24 m	*Ad Libitum* Caloric restriction	MWM	Positive effects
Algeri et al., 1991	Sprague-Dawley derived CD-COBS	4 m 12 m 24 m	*Ad Libitum* Caloric restriction	Passive avoidance task	Positive effects
Algeri et al., 1991	Sprague-Dawley derived CD-COBS	12 m 24 m 30 m	*Ad Libitum* Caloric restriction	MWM	Positive effects
Pitsikas & Algeri, 1992	Sprague-Dawley derived CD-COBS	3 m 11 m 25 m	*Ad Libitum* Hypocaloric diet	8 arm radial maze MWM	Positive effects
Gyger et al., 1992	Sprague-Dawley	6m 12m 19m 24m	*Ad Libitum* Intermittent fasting Caloric restriction	MWM	Positive effects of CR in test and reversal
Markowska, 1999	Fischer 344 Brown-Norway	6 m 12 m 18 m 24 m	*Ad Libitum* Caloric restriction	MWM	No effects
Markowska & Savonenko, 2002	Fischer344xBrown- Norway Brown-Norway	9 m 18 m 30 m	*Ad Libitum* Caloric restriction	MWM	Positive effects in Fischer344xBrown- Norway
Yanai et al., 2004	Wistar	12m 17m	*Ad Libitum*	MWW DMTP	Positive effects

			Caloric restriction		
Hansalik et al., 2006	Sprague-Dawley	5m 10m 18m	*Ad Libitum* Caloric restriction	MWM	No effects
Geng et al., 2007	Sprague-Dawley	18 m	*Ad Libitum* Caloric restriction for 6 months	MWM	Positive effects
Fitting et al., 2008	Fischer344xBrown Norway	3 m 36 m	*Ad Libitum* Caloric restriction	MWM	Slight positive effect on acquisition
Adams et al., 2008	Fischer344xBrown Norway	10-12 m 18-20 m 29-32 m	*Ad Libitum* Caloric restriction	MWM	Positive effects
Carter et al., 2009	Fischer344xBrown Norway	8 m 12-15 m 25-27 m 35-38 m	*Ad Libitum* Caloric restriction	Object recognition MWM	Positive effects in MWM
Del Arco et al., 2011	Wistar	6m 15m 24m 30m	*Ad Libitum* Caloric restriction	Alternating delayed task in T water maze	No effects
Singh et al., 2015	Wistar	3m 15m	*Ad Libitum* Intermittent fasting	Rotarod	Positive effects

2.3.2 Studies in mice

Fewer investigations into aging and CR in mice have been carried out but the results are more homogeneous. Moreover, all the experiments have used same strain of animals, the C57BL6N, with the exception of one carried out with the C3B10RF1 strain in a radial maze, which showed positive effects of CR on age-related memory (Ingram et al., 1987). In general, the behavioral tasks employed have been spatial memory tasks such as MWM (Bellush et al., 1996; Dong et al., 2016; Fann et al., 2014; Guo et al., 2015; Kuhla et al., 2013; Meas et al., 1993; Parikh et al., 2016;

Xu et al., 2015) and radial mazes (Guo et al., 2015; Idrobo et al., 1987; Parikh et al., 2016). On the whole, results obtained seem to confirm the beneficial effects of CR on memory in aged mice (Table 6), with the exception of one investigation (Means et al., 1993), in which 22 to 25 month CR old mice did not show differences in MWM performance when compared with a 13 month old control group, as well as the results from another study (Bellush et al., 1996) in which CR animals performance in escape latencies in the MWM did not differ from young controls.

Table 6: Different experiments over time that studied the effects of CR in learning and memory of aged mice.

Author	Strain	Age (months)	Intervention	Task	Results
Idrobo et al., 1987	C57BL6N	15m	*Ad Libitum* Caloric restriction	Radial arm maze	Positive effects
Ingram et al., 1987	C3B10RF1	11-15 m 31-35 m	*Ad Libitum* Caloric restriction	Complex maze	Positive effects
Means et al., 1993	C57BL6N	13m 22m 25m	*Ad Libitum* Caloric restriction since 14-month-old	MWM	No effects
Bellush et al., 1996	C57BL6N	4 m 19 m	*Ad Libitum* Caloric restriction	MWM	No effects
Kuhla et al., 2013	C57BL6N	2 m 6 m 20 m	*Ad Libitum* Caloric restriction	MWM	Positive effects in 20 month old group
Yang et al., 2014	C57BL6N	3 m 12 m 20 m	*Ad Libitum* Caloric restriction	MWM	Positive effects
Guo et al., 2015	C57BL6N	5-6 m 18-20 m	*Ad Libitum* Caloric restriction	Radial Maze MWM	Positive effects
Dong et al., 2016	C57BL6N	12 m	*Ad Libitum* Caloric restriction Caloric increase	MWM	Positive effects
Xu et al., 2015	C57BL6N	6 m	*Ad Libitum* Caloric restriction	MWM	Positive effects with CR

			Resveratrol supply		
Parikh et al., 2016	C57BL6N	6m 20m	*Ad Libitum* Caloric restriction	Radial Maze MWM	Positive effects

2.3.3 Studies in humans

Effects of CR in the human population

In general, investigations into the effects of CR diet on human cognitive processes are very scarce. Most studies have evaluated either the effects of diets with high caloric content or the intake of certain nutritional components (Swaminathan & Jicha, 2014). In these cases, a high fat diet has been linked to a higher risk of age dependent dementia (Gillette-Guyonnet et al., 2013; Morris & Tangney, 2014; Murphy et al., 2014).

The benefits of a healthy Mediterranean diet for cognitive process in humans have been reported in one study (Smyth et al., 2015) that involved thousands of elderlies (over 55 years old) people from 40 different countries for five years. The results concluded that people who followed a healthy Mediterranean diet had less risk of cognitive decline during the five-year study. Carrying out a CR experiment on the human population involves many difficulties and challenges. Nevertheless, it has been shown that 30% CR was able to improve memory retention in healthy older people compared to a regular diet or a diet that included omega 3 supplements. The results seem to confirm the laboratory studies in rodents. The CR group presented significantly better verbal memory than the other groups.

Another interesting study to assess the effects of CR in humans was carried out in 2007 by the National Institute on Aging (NIA). The CALERIE program (Comprehensive Assessment of Long-Term Effects of Reducing Calorie Intake) studied 220 adults on a 25% CR diet program for two years (Rickman et al., 2011). The results indicated that this percentage of CR decreases the risk of cardiovascular disease in humans (Lefevre et al., 2009) and reduces DNA damage by improving mitochondrial function (Civitarese et al., 2007). The CALERIE program had a preliminary attempt in which the subjects had to follow the same instructions, however, it failed because it was revealed that the percentage of CR was only 10% because the participants were not following the instructions (Racette et al., 2006). This concern is still a problem, humans find it difficult to maintain a CR diet and the number of calories and thus the results can be imprecise (Roth & Polotsky, 2012). Along similar lines, one of the biggest problems associated with carrying

out CR experiments in humans is the evaluation and examination of the intake of the *Ad Libitum* group. When unlimited food is provided to subjects they consume more food than the basal and predicted intake (Arslan-Ergul et al., 2013). In addition, the control group tend to be more sedentary, obese and glucose intolerant compared to the CR group (Vaughan et al., 2017).

The future of the CR investigation in humans: Mimetics

In general, 25% to 30% CR in humans is a reduction that requires much less food consumption than expected. It can be difficult for humans to complete the diet, and can even lead to depression (Arslan-Ergul et al., 2013). Therefore, the development of CR mimetics (CRM) is the future of investigation in this field in humans (Ingram et al., 2006). The ideal CRM would be an agent consumed in food or water that would mimic the metabolic, hormonal and physiological effects of CR, delaying death and age-associated diseases without requiring a change in calorie intake. Ten years ago, the main focus was 2-deoxy-glucose and other glycolysis inhibitors. However, further investigation revealed that this compound produced cardiotoxicity in rats at the doses used (Ingram & Roth, 2015). Nowadays, the strategies proposed are those that blocks mTOR in the nutrient-signaling pathway (Arslan-Ergul et al., 2013). In addition, further proposals are trying to target Insulin receptors to reduce plasma levels of insulin, which has been shown to be a predictor of longevity in healthy humans (Roth et al., 2002). Another approach is related to the action of IGF-1, but no strong candidate CRM for IGF-1 has yet emerged. However, an antagonist of the GH receptor that reduces the production of IGF-1, which is being used for acromegaly, *Pegvisomant* is being investigated (Kopchick et al., 2014) but results from this CRM are yet to be reported. Progress is needed in these various strategies to use them as a CRM in humans in the future.

IV. METHODS

IV. METHODS

1. Subjects

Eighty-two naive male Wistar rats (Figure 3), obtained from our laboratory breeding stock (Prolabor, Charles River Laboratories, Arbresle, France) were used. The animals were separated to be used in two different experiments, which consisted of three groups each. Specifically, we used a Young group (n= 28; age = 3-4 mo; weight = 481.48 gr; SEM= 9,70) with free access to food and water, an *Ad Libitum* old group (n= 23; age = 24-27 mo; weight = 667.85 gr; SEM= 20,39 gr), with free access to food and water, and a CR old group, (n= 31; age= 24-27 mo; weight = 502.52 gr; SEM = 8,48 gr) with a 25-30% reduction in food intake from the age of 4 months old, and which followed the food restriction procedure described in Speakman and Mitchell (2011). The percentage of food reduction was previously studied by analyzing the amount of food that an adult Wistar rat eats in the situation of free access to consumption. The young paired animals ate 52.63 gr per day, while the *Ad Libitum* animals 37.95 gr of food. A mean from both groups was calculated and a 25-30% of the result was extracted from the CR group's daily food allowance. The paired CR animals ate around 30-34 gr of food per day, 15 to 18 gr of food for each rodent and free access to water were given to CR animals from the age of 4 months old to aging.

Figure 3: Three subjects. From left to right, a CR aged rat, a young rat and an Ad Libitum aged Wistar rat.

The animals were fed with dry pellets from Harlan Laboratories; Inc. (Madison, USA) produced and packed by Mucedola, Sri. (MI, Italy). It consisted of a Global diet (2014), with a composition of: Wheat, Maize, Maize gluten, Calcium carbonate, Soybean oil, Mineral dicalcium phosphate, Corn gluten feed, Sodium chloride, and Magnesium oxide. Additives (per kg): Vitamin A (E672) 6000 I.U, Vitamin D3 (E671) 600 I.U, Fe (E1) 50 mg, Mn (E5) 44 mg, Zn (E6) 31 mg, Cu (E4) 7mg,

I (E2) 6.2mg, Co (3b302) 0.5mg, and with the following analytical constituents: Moisture12%, Crude protein 14.5%, Crude oils and fats 4%, Crude fiber 4.5%, and Crude ash 4.7%.

All throughout the experiment the animals were paired housed in 50 x 22 x 14 cm transparent plastic sawdust-bedded cages (Figure 4). All subjects were maintained in a controlled environment of humidity (60-70%), temperature (20ºc to 22ºc) and on a 12-hour light-dark cycle (light from 7 to 19, dark to 19 to 7). All the behavioral experiments were performed during the light cycle, between 8 am and 13 pm.

Figure 4: Image of the facilities where the animals are estabulated during the aging and the experimental process. The rack presents a group of young and CR animals paired housed. Laboratory of Psychobiology, UAB.

Ethics and animal welfare

All procedures were performed following the EU Directive on the protection of animals used for experimental and other scientific purposes (86/609/EEC) and with the authorization of the Generalitat de Catalunya (DOBC 2450 7/8/1997, DAGP number 3046). Moreover, the experiments were approved by "*Comissió d'Ètica en l'Experimentació Animal i Humana (CEEAH)"* of the Autonomus Unviersity of Barcelona (DARP, protocol number 8694).

2. Behavioral tasks

2.1 Open Field

OF exploration test measures the ability to systematically assess novel explorations of the environment, the general locomotor activity, and it also provides an initial screen for anxiety-like behavior in rodents. The behavioral assessment of the animals in the OF test was performed on the basis of previously described methodology (Márquez et al., 2002). A single session was accomplished 3 to 5 days before the following behavioral task were completed. The OF maze consisted of a wall-enclosed circular area of 80 cm diameter with walls of sufficient height, 34 cm, to prevent the subject from escaping. A camera was mounted above the maze (JVC, Everio Model GZ-X900), and connected to a monitor in order to record each session and analyze the variables using computer software (Smart Video Tracking System, Version 3, Panlab, Barcelona, Spain) (Figure 5). A white light bulb of 50W was set above the maze, near the camera. During all the trials, a background sound was turned on to avoid possible distractions.

Figure 5: Image of the OF structure of the laboratory of psychobiology (UAB).

The maze was divided into three different zones (Figure 6): the central area (zone 1) with 21 cm of diameter, was defined as the OF center, the medium area (zone 2) was around the center, with a width of 15 cm and the peripheral zone, near to the walls (zone 3), consisted of 11 cm width. The animal was placed into the maze facing the wall, always in the same spot, and then allowed to move freely in the area for 10 min. The apparatus was cleaned with 70% ethanol after each animal and for subsequent tests in order to remove any scent clues left by the previous subject.

The variables permanency at each zone, mean speed and number of entrances to each zone were analyzed. Several examples of traces of locomotor activity are presented in the Figure 6.

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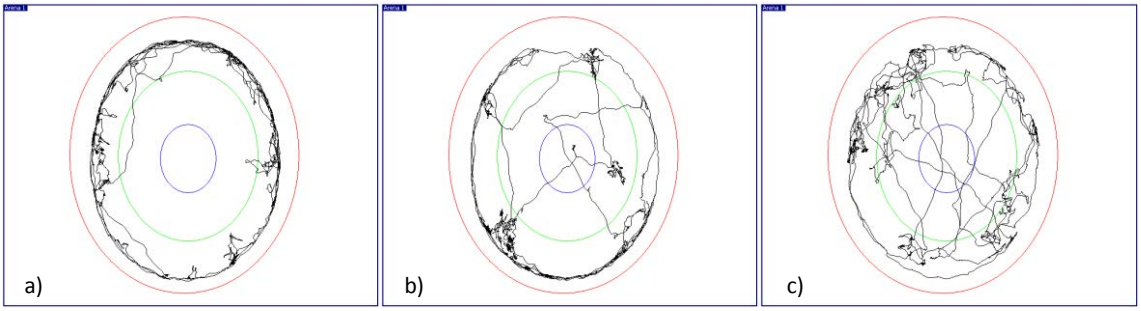


Figure 6: Examples of locomotor activity during the OF task. The a) pathway belongs to an Ad Libitum aged rat, b) to a CR aged animal and c) to a young Wistar rat.

2.2 Elevated Plus Maze

Anxiety-like behavior levels were evaluated using the EPM (Pellow et al., 1985). A single session was performed 2 hours after the OF. The EPM consisted of a black plastic structure with four arms (50 x 12 cm) forming a cross from a neutral central square (12 x 12 cm) (Figure 7). Two of the arms were protected by vertical walls (closed arms) of 40cm high, while the other two perpendicular arms had unprotected edges (open arms), adapted from (Walf & Frye, 2007). The maze was raised 50 cm above the floor level and illuminated from above (50 W). During all the trials, a background sound was turned on to avoid possible distractions.

Figure 7: Image of the EPM structure provided in the laboratory of psychobiology (UAB).

The EPM experiments were conducted as previously reported (Walf & Frye, 2007). Briefly, each rat was placed in the central neutral area at the beginning of the 5 min observation session, facing one of the open arms. The time spent in each arm started to count when the animal moved its head and the two forepaws into the arm. The percentage of time spent in the closed arms of the EPM was considered as a measure of anxiety-like behavior. The total number of entrances to each arm, and two different anxiety ratios (total time in open arm/total time of the task and number of open arm entries/total number of entries) were analyzed. After each trial, the apparatus was cleaned with 70% ethanol to avoid odor traces.

2.3 Morris Water Maze

The MWM was developed by Richard Morris in 1984. It consists of a test of spatial learning that relies on distal cues to navigate in an open swimming area from a start location around the perimeter to a submerged hidden escape platform (Morris, 1984). The spatial learning is assessed across the repeated trials and memory is determined by the preference for the area surrounding the target when the platform is absent. The MWM consisted of a circular black wall pool (2 m diameter, 60 cm above the floor) filled with 45 cm of water and maintained at a temperature of around 22 ± 2 degree Celsius. The pool was placed in a dark room and it was surrounded by a black curtain forming a circular enclosure of 2.4 m in diameter. In the inside face of the curtain, facing the pool, there were visual cues to help the rat locate the platform (Morris, 1984). The animals had to escape from the water to a small black platform (11 cm of diameter), which was hidden under the surface (2 cm deep from the water). The location of the platform could only be found by searching for the distal visual cues surrounding the pool, which were a plastic beach ball with different colors in vertical like blue, white, yellow and orange, a white box with stripes painted in black, a stuffed animal (teddy bear) and a region with a potent light facing the curtain (Chamorro-López et al., 2012). A graphic representation of the MWM with the visual clues is shown below (Figure 8). During the different trials, the swim path of the animal was tracked and recorded using a video camera connected to a pc running a specific program (Smart Video Tracking System, Version 2.5, Panlab, Barcelona, Spain).

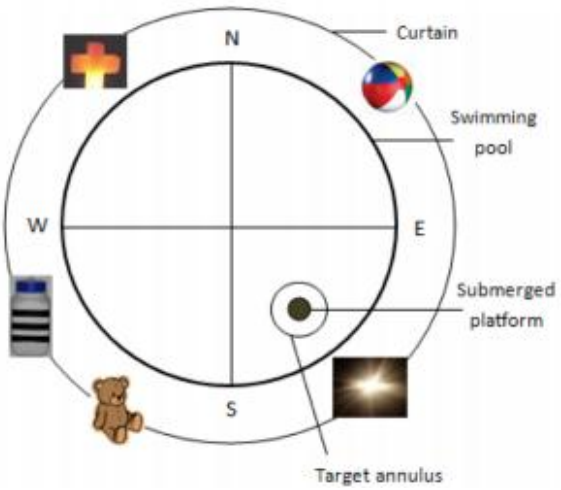


Figure 8: Graphic representation of MWM with the visual cues.

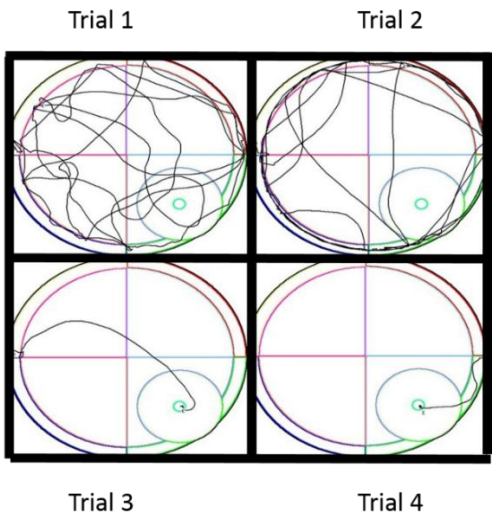


Figure 9: Examples of swim paths during an acquisition session in the MWM.

Acquisition phase

The acquisition consisted of five consecutive days with four trials of 90s each day, and the intertrial interval was 120s (Figure 10). In each trial, the animals were placed into the water facing the wall of the pool from one of four cardinal points: North (N), South (S), East (E) or West (W), which were chosen randomly. They were required to find the platform, which was located in the SE quadrant in every trial. If the animal found the platform, it was allowed to remain on it for 15s and was then removed from the pool by the experimenter. If the animal failed to find the hidden platform within the 90s of the trial, it was manually guided by the experimenter to the platform and allowed to stay on it for 15s before being removed from the pool. During the intertrial time, the animal was placed inside a metal box with paper and constant hot temperature, to help recover. The latency to find the platform, swim speed, time at the platform quadrant, total length (total distance swim) and *thigmotaxis* (time spent near the walls) were analyzed. A graphic representation of the swim paths is shown (Figure 9).

Probe Test and Reversal Learning phase

Seventy-two hours after the last acquisition trial the platform was removed from the pool and the animal was placed inside the water from the East (E) cardinal point. The length of the trial was 60s time in which the animal was allowed to swim freely, while trying to find the non-existent platform. During the probe test phase, the percentage of time spent in the target quadrant (time spent in the quadrant where the platform was, versus the time spent in the other quadrants), and in the target annulus (the area surrounding the platform's location in the previous phase), swim speed, total length travelled, *thigmotaxis,* and number of target crossing were analyzed. After the test trial was over, the experimenter inserted the platform in a new position in order to begin with the reversal learning phase, which evaluated whether or not the animal could inhibit the initial learning and which requires the involvement of a flexible cognitive map (Vorhees & Williams, 2006). The platform was placed in the opposite quadrant in reference to the previous location (the platform was situated in the SE quadrant during the acquisition, while it was situated in the NO quadrant for the reversal phase). The experimenter guided the animal to the platform and let it mount it for 15s before removing the animal from the pool and placing it in the metal cage for the intertrial time of 120s. Then, three more trials were performed using the new position of the platform (NO), (Figure 10) in which the cardinal point to introduce the animal was randomly changed across trials. Parameters of mean latency to find the new location of the platform, swim speed, length and *thigmotaxis* were analyzed.

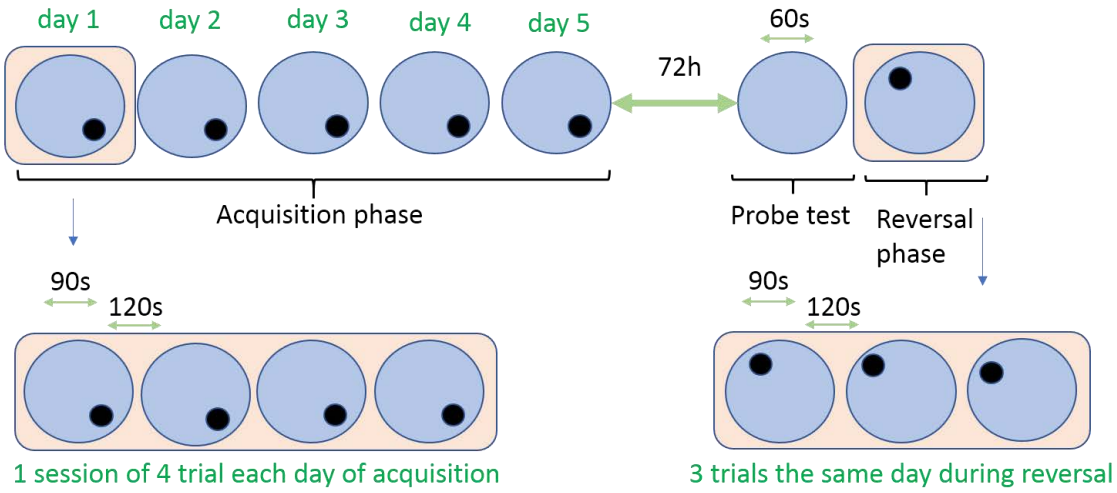


Figure 8: Schematically draw of the three different phases of the MWM in time line.

2.4 Odor Discrimination Task

The ODT consists of a simple associative learning task, in which animals use olfactory signals to carry out the learning process and it is based on the innate and natural tendency of animals to explore new stimuli and to discriminate them from known, familiar ones. The ODT is quick to acquire and the memory is easily maintained over time (Sara et al., 1999). The task was performed in a black square box (60 x 60 x 40 cm) containing three yellow sponges (8.5 x 6.5 x 5.5 cm) with a 3 cm diameter hole cut into the center to a depth of 2.5 cm. The sponge was placed in a glass slide holder of the same size. The food reinforcement was placed in the hole of the sponge. The reinforcement consisted of a crispy chocolate rice breakfast cereal (Kellogg's, Spain). Each sponge was infused with an odor, vanilla (0.3 ml), orange (0.6 ml) and anise (0.2 ml) (Vahiné, Ducros S.A. Sabadell, Spain) that were injected in each of the corners of the sponge. All the aromas were tested in a previous pilot study, in which the animals showed no preference. The sessions were recorded by a video camera (JVC, Everio Model GZ-X900) connected to a monitor. A white light bulb of 50W was installed in the ceiling near the camera. The following procedures are based on the experiments by Sara and colleagues (Sara et al., 1999).

Habituation sessions

All the rats were food-deprived for five to eight days in order to achieve a final mass of 85-90% of the initial weight prior to the three habituation sessions, in which they were given free access to the food reinforcement (crispy chocolate rice breakfast cereal) for three consecutive days. The habituation was performed in an opaque plastic bottomed cage (50 x 22 x14 cm) and the time that it took the animals to consume 10 pieces of the reinforcement, the chocolate cereal, was measured. After consuming the reinforcement, each animal was placed in the training box,

which consisted of a black square box with three sponges placed in an L-shaped configuration, without reinforcement. The ODT was performed in the latter configuration, which the animals were allowed to explore for 15 min. The time spent eating the food reinforcement was recorded.

Acquisition session

One day after the last habituation session, ODT was carried out in a 4-trial session (Figure 11), as described in previous procedures (Quiroz-Padilla et al., 2007). The reinforcement (unconditioned stimulus, crispy chocolate rice breakfast cereal) was associated with the same odor across trials (conditioned stimulus). This condition was randomly assigned to each rat and counterbalanced within the different groups. The sponges with the non-reinforced odors did not contain food. The sponges were placed in three of the four corners of the black box and the position of each sponge infused with the odor was changed for each trial of the acquisition phase according to previous protocol. At the beginning of the acquisition session, the rats were placed in the black box, facing the corner with no sponge. When the animal found and ate the cereal they were removed and placed in an intertrial cage (opaque plastic box 50 x 22 x14 cm) for 1 min. The procedure was carried out again for a total of four trials; each trial consisted of a 3 min maximum period for the rat to find the reinforcement. The failure to find the crispy chocolate resulted in the rat being removed to the intertrial box for 1 min before the next trial. Two different types of errors were recorded: omission error (sniffing the rewarded sponge with no subsequent nose-poking) and commission error (nose-poking into a non-rewarded sponge) (Tronel, 2002).

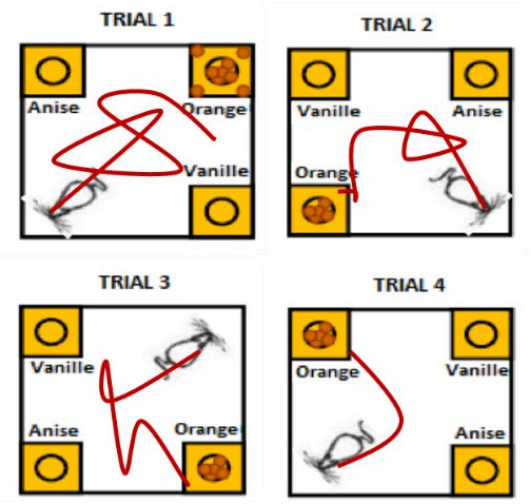


Figure 11: Schematic representation of the acquisition phase in the ODT.

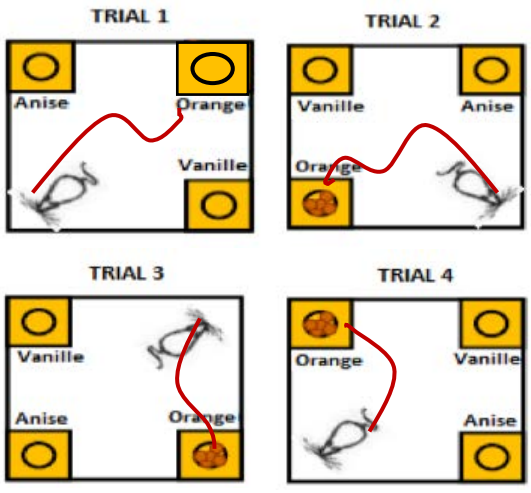


Figure 12: Schematic representation of the test (trial 1) and reacquisition trials in the ODT.

Test and reacquisition sessions

Seventy-two hours after acquisition, a test session was carried out (Figure 12), in which the animals were introduced in the same acquisition black box but, this time, the reinforced sponge contained no chocolate cereal. The trial was considered completed when the rat had performed the correct response, nose-poking into the previously rewarded infused sponge. Quickly after the animal showed the response, it was removed from the box to prevent the extinction of the response. There was 1 min intertrial period between trials. The other 3 trials were exactly the same as in the acquisition session, in which the originally rewarded odor was again associated with the cereal. There was a maximum of 3 min exploration time, after which the animal was removed from the box. The latency to find the rewarded sponge and start eating the reinforcement, as well as both types of errors (omission and commission) were registered and analyzed.

2.5 Olfactory Perception Test

After the ODT, in order to rule out olfactory impairments due to aging, an additional olfactory perception test was conducted. Twenty-four hours prior to the olfactory test, the rats were feed in the housed cage (in pairs) with a butter-flavored cookie (Brambly Hedge, Denmark). Their access to normal food pellets was restricted until the olfactory perception test took place. The following day, the test was conducted as described in previous reports (Portero-Tresserra et al., 2013; Wrenn et al., 2003). A clean plastic rat cage was set up (50 x 22 x 14 cm), filled with 2 cm of sawdust. One butter-flavored cookie was hidden and covered with sawdust in one of the corners. The position of the cookie was randomized to each animal. The rat was placed in the opposite corner from the buried cookie. The latency to find the buried cookie and commence eating it was recorded and analyzed. The sawdust was changed and the walls of the cage were cleaned with 70% ethanol after testing each animal to prevent potential odor trace.

2.6 Object Recognition in Y maze

The first object recognition task was carried out by Ennaceur and Delacour (1988), for a neurobiological study of memory in rats without the need to learn a rule. It was based in the presentation of two familiar objects in the first trial and then, in the second trial, the familiar object was change for a novel one. Novelty is an alteration from an expected event on the basis of previous information and internal estimates of probabilities (Antunes & Biala, 2012) that can affect animals by changing their behavior, triggering stress response and eliciting approach behavior to the novelty (Bevins et al., 2002). Object recognition test can be performed in a Y

maze in which its spatial component, recognition of the location and direction of the arms, is considered a hippocampal-dependent learning task (Conrad et al., 1996). However, other brain regions may be acting in the object-recognition learning, which means that it could not only be dependent on the HPC (Mumby et al., 1992). For this reason, object recognition performed in a Y maze is considered both a prefrontal and hippocampal dependent task.

Figure 13: Image of the two kinds of objects used during the object recognition with Y maze.

The apparatus was constructed of three black plastic arms (45 x 15 x 40 cm), as an adaptation based on previous reports (Dellu et al., 1992). An object was placed at the end of each arm. The objects were a soda can (11.5 x 7 cm), or a Lego built inverted T shape (6.5 x 10.5 x 3.5 cm) (Figure 13). The sessions were recorded with a camera (JVC, Everio Model GZ-X900) and analyzed using software tracking (Smart Video Tracking System, Version 3, Panlab, Barcelona, Spain). In order to minimize the odor trace of the animals, the apparatus was cleaned with ethanol 70% and paper after each trial. The maze was located in a room, and a white 50W light bulb was placed above the maze. Numerous distal visual cues (walls, air-conditioned machine, curtains and posters) were located around the Y maze in order to facilitate the orientation of the animals; those were kept constant during all the behavioral period. Prior to conducting the test of object recognition, a pilot study in ten three-month old rats was performed in order to determine if the animals showed preference for a particular arm or object. The time exploring each arm and each object was analyzed (Results: Y maze, previous preference test).

Habituation session

Twenty-four hours before the test was performed, all animals were able to explore the maze. Two habituation trials of 15 min each with an intertrial of 30 min were performed. One hour before the acquisition, the animals were kept in a room with the same temperature, light and noise, conditions as the maze room. The animal was introduced in the maze from one of the three arms, the initial arm (the same arm for all the behavioral testing) facing the wall. The rat had 15 min to explore the maze freely. All the trials were recorded and variables such as first choice (first visited arm) and the number of entrances to each of the arms were registered by the experimenter (Dellu et al., 2000). After the first trial of habituation, the animal was returned

to its cage and paired with their partner for the 30 min of intertrial time. Meanwhile, the maze was cleaned with 70% ethanol and paper. After the intertrial time, the animal was introduced into the maze again for a further 15 min of free exploration.

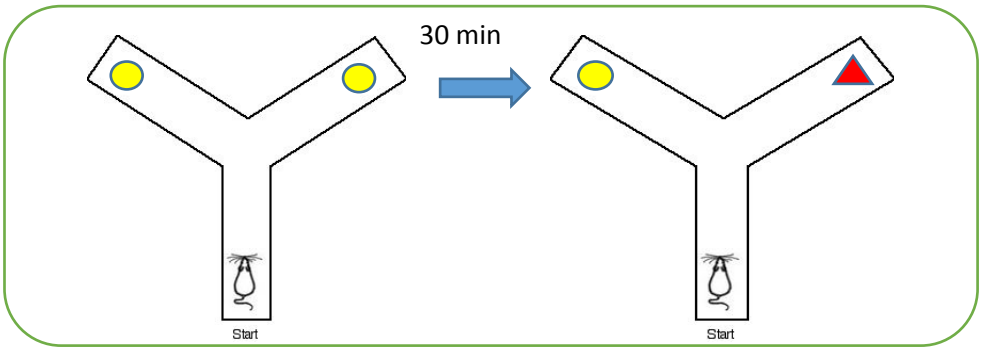


Figure 14: Schematic representation of the object recognition test in the Y maze. Familiar object is represented with yellow dot and novel object with red triangle.

Object recognition test phase

Twenty-four hours after the last habituation trial, two trials of 15 minutes of free exploration and an intertrial session of 30 minutes were carried out, but this time the first trial included two objects at the end of the two arms (Figure 14). In the first trial, two identical objects were placed at the end of the arms. In the second trial, an identical copy of the object used for the acquisition session (familiar object) and a new object were placed at the end of the maze arms (Figure 15). The familiar and/or novel objects, as well as their locations, were used in a balanced manner to reduce potential biases due to potential preferences for a particular location or object. The first choice (first visited arm), the time exploring in each arm and the exploration time of the familiar and the novel object were recorded and analyzed. Once the test was finished, the maze was cleaned with 70% ethanol.

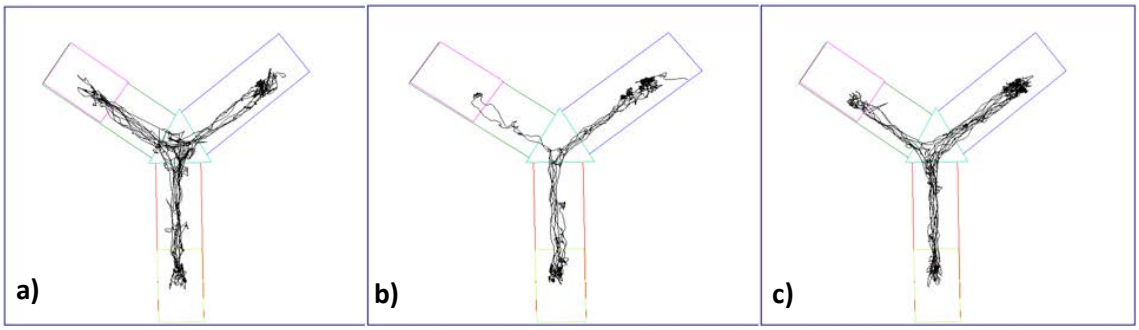


Figure 15: Examples of the trajectory of the animals in the test phase of the object recognition in the Y maze. a) Ad Libitum old rat b) Young rat c) CR old rat.

3 Experimental design

The OF and EPM, which were used as locomotor and anxiety control tests, were performed at the beginning of both experiments, using a subsample of subjects of each group. In experiment 1, the order of presentation of the two tasks, MWM and ODT, was counterbalanced to prevent interference from one task to the other. The first group performed the MWM test first and then the ODT, the second group performed the tasks in the opposite order (Figure 16). Experiment 2 was just composed of one behavioral task, object recognition in the Y maze, therefore no counterbalance of the tasks was necessary.

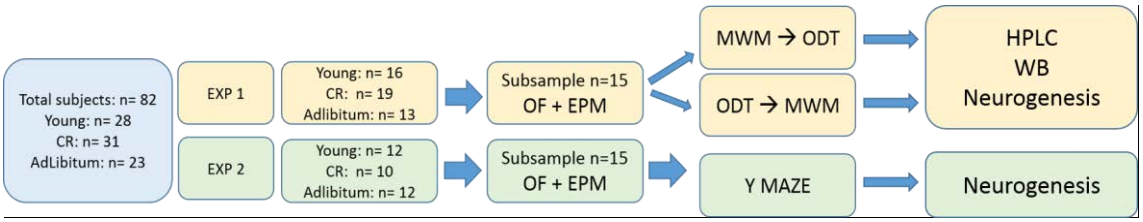


Figure 16: Experimental design for the behavioral tasks. EXP 1 was carried out first and EXP 2 second. Abbreviations: EXP (experiment) OF (Open Field) EPM (Elevated plus maze) MWM (Morris Water Maze) ODT (Simple Odor discrimination task) HPLC (High performance liquid chromatography) WB (Western Blot).

4 Biochemical procedures

4.1 Sample acquisition

After completing all the behavioral procedures from the experiment 1, half of the animals were kept on a fasting condition for 24 hours and then euthanized by decapitation with a manual guillotine. Blood samples were collected from the jugular vein and the carotid artery of the neck for a later analysis of the plasma, in tubs containing Heparin (Sodium Heparin, 5000 USP/mL; Chiesi Spain, SA, Spain) to avoid the coagulation. Blood samples were placed on ice for slow coagulation and were centrifuged at 4000rpm for 15 min at 4ºC to obtain cell-free plasma, which was stored at -20ºC until biochemistry analysis. Afterwards, the brain was rapidly removed and dissected on ice into nine regions that were weighed, frozen and stored at -80ºC. The following brain regions were collected separately: cerebellum, protuberance, midbrain, HT, thalamus, HPC, striatum, FC and occipital cortex. Moreover, the HPC, striatum and FC were divided into 2 equal pieces for a posterior use (Methodology section 3.4.2 and 3.4.3, WB and HPLC).

The other half of the animals from the experiment 1 and all the samples from the experiment 2 were dispensed a lethal dose of sodium pentobarbital (Dolethal, 200 mg/kg; Vetoquinol SA, Madrid, Spain) in 1 ml + 1 ml of physiologic serum. Once the animal had lost the palmar reflex,

an intracardiac perfusion was performed in order to rapidly and uniformly fix the brain and preserve the tissue in a life-like state. For the perfusion, PBS (phosphate-buffered saline 0.1M, pH 7.4) and PFA (paraformaldehyde at 4% dissolved in PBS) were used. The brain was then removed and submerged in PFA for three hours, after which it was. Then, three more washes of 20 min each with PB (phosphate buffer) were performed. Finally, the brain was submerged in a 30% sucrose cryoprotective solution in PB for 72h. In order to preserve the tissue, the brains were frozen using liquid isopentane (320404, Sigma-Aldrich, US) for 2 min, in dry ice, until the temperature was between -40ºC and -60ºC. All the brains were stored at -80ºC for posterior analyses (data not shown).

4.2 Western Blotting

Sample preparation

Samples from the HPC, the striatum and the FC were collected in lysis buffer (0.15M NaCl, 1% TX-100, 10% Glycerol; $C_3H_8O_3$; 0.001M EDTA; $C_{10}H_{16}N_2O_8$, 0.05M TRIS, $(HOCH_2)_3CNH_2$; pH 7.4). The buffer contained phosphatase inhibitors (04906845001, Roche, France) and protease inhibitor tablets (05892970001, Roche, France). The lysates were homogenated manually using Wheaton™ Dounce Tissue Grinders (ThermoFisher Scientific, USA), 20 times with the Loose and 20 times with Tight grinder. Lysis buffer was used as a homogenated buffer. The concentration in relation to the weight of each cerebral region was x15 in the samples of FC and x30 in the samples of HPC and striatum (Table 7). After the samples were manually homogenated, they spent 20 min in a 4ºC centrifuge at 4000 rpm in order to separate the pellet from the supernatant that was storage in 1 ml Eppendorfs at -20ºC. The total amount of protein was quantified using Pierce™ BCA assay kit (Pierce Chemical Co., Termofisher, USA). The samples with the reactives (50 µl Reagent A / 1 µl Reagent B) from the kit were incubated in a 96 well plate for 30 min at 37ºC, following the manufacture instructions, and the absorbance of each sample in 562 nm was measured with the plate reader BIO-TEK Power Wave XS Spectrophotometer (BioTek Instruments, USA). The final concentration of each sample was calculated from the extrapolation with the straight pattern, of Bovine Serum Albumin, which was included in the BCA kit. The quantification of FC samples was diluted 1:10 with purified water and the concentration was 3 ug/uL, the striatum samples were diluted 1:5 with purified water and the final concentration was 3 ug/uL and the HPC samples were diluted 1:10 and the final concentration was 7 ug/uL.

Table 7: Weight of each cerebral area (mg) and the lysis buffer added to each subject area, hippocampus and striatum were in a proportion of x30 about the area weight and frontal cortex x15.

Subject identification	Hippocampus weight (mg)	Lysis Buffer added (μL)	Striatum weight (mg)	Lysis Buffer added (μL)	Frontal lobe weight (mg)	Lysis Buffer added (μL)
CR_1	67.2	2016.0	40.0	1200.0	258.0	3870
CR_2	87.0	2610.0	42.7	1281.0	209.3	3139.5
CR_3	65.2	1956.0	45.5	1365.0	345.8	5187
CR_4	93.4	2802.0	44.7	1341.0	310.1	4651.5
CR_5	81.0	2430.0	48.8	1464.0	285.3	4279.5
CR_6	113.2	3396.0	46.5	1395.0	296.7	4450.5
CR_7	88.6	2658.0	31.2	936.0	294.8	4422
CR_8	85.7	2571.0	63.7	1911.0	287.3	4309.5
CR_9	73.4	2202.0	22.1	663.0	285.5	4282.5
CR_10	60.4	1812.0	36.2	1086.0	325.4	4881
AdLib_-	65.9	1977.0	19.4	582.0	260.5	3907.5
AdLib_0	83.0	2490.0	141.0	4230.0	301.1	4516.5
AdLib_5	56.6	1698.0	85.3	2559.0	285.0	4275
AdLib_6	59.8	1794.0	69.9	2097.0	228.1	3421.5
AdLib_7	80.4	2412.0	90.0	2700.0	236.9	3553.5
AdLib_8	84.9	2547.0	75.9	2277.0	194.0	2910
AdLib_9	102.8	3084.0	79.4	2382.0	278.7	4180.5
AdLib_10	66.9	2007.0	69.7	2091.0	261.5	3922.5
Young_1	42.8	1284.0	78.4	2352.0	239.7	3595.5
Young_2	46.5	1395.0	48.2	1446.0	237.8	3567
Young_5	81.7	2451.0	49.2	1476.0	252.8	3792
Young_6	68.0	2040.0	50.3	1509.0	243.9	3658.5
Young_9	84.0	2520.0	55.3	1659.0	220.0	3300
Young_10	58.8	1764.0	57.4	1722.0	178.1	2671.5
Young_13	49.6	1488.0	38.6	1158.0	222.0	3330
Young_14	36.5	1095.0	47.8	1434.0	134.1	2011.5

Once the total protein concentration from the lysate was measured and determined, the x4 Loading Buffer (LB) (60 mM Tris-HCl at pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol and 0.01% bromophenol blue) was prepared. It contained β-mercaptoethanol, to reduce the intra and intermolecular disulfide bonds, sodium dodecyl sulfate (SDS) a detergent to denature the proteins, bromophenol blue, dye agent that makes it easy to see the sample during loading, and glycerol, which increases the density of the sample. The total mix was composed of: 50 μL of 4x LB, a proportion of lysis buffer with the subject sample dependent on the concentration of each sample, and finally brought up to 200 μL using 1x LB. The samples were then boiled at 95ºC for 5 min to ensure the protein denaturation.

Gel preparation and protein transfer

Gels were prepared manually between two glass plates (Mini-PROTEAN Spacer Plates, Bio Rad, Hercules, CA, USA), short plate (10.1 x 7.3 cm), and spacer plate (10.1 x 8.2 cm of 1.5 mm in thickness) and charged with 30 μg of protein in LB in a 12% SDS-PAGE gel. The process started with the polymerization of the separating gel, with a 12% of polyacrylamide (Table 8), followed by the pouring of the stacking, gel with a 3.5% of polyacrylamide, which had a comb inserted at the top to create the sample wells (10 or 15 wells).

*Table 8: Composition of the gels of SDS-PAGE for a 1.5 mm of thickness gel. *B solution is composed of SDS 0.4% and Tris-HCl 1M at pH 8.8, and **C solution is composed of SDS 0.4% and Tris-HCl 0.5M at pH 6.8.*

Separating gel 12% PAA	**Stacking gel 3.5% PAA**
Acrilamide (mL) 3	Acrilamide (mL) 0.7
B Solution* (mL) 1.875	C Solution** (mL) 1.5
H20mQ (mL) 2.625	H20mQ (mL) 3.8
TEMED (μL) 100	TEMED (μL) 10
APS 10% (μL) 100	APS 10% (μL) 100

For the electrophoresis, four gels were inserted in a Mini-PROTEAN Tetra Electrode Assembly (Bio Rad, Hercules, CA, USA) into the tank of Mini-PROTEAN Tetra Cell for Ready Gel Precast Gels (Bio Rad, Hercules, CA, USA), and 30 μg of protein sample was loaded in each well. In addition, 4 μL of molecular weight marked was loaded to one well of each gel (Precision Plus Protein Dual Color Standards; Bio-Rad, USA). The tank was filled with running buffer composed of 25 mM Tris-HCl, 192 mM glycine, 3.5 mM SDS. The electrophoresis lasted for 90 min and was set at 120V a PowerPax HC Power Supply (Bio-Rad; Hercules, CA, USA).

The proteins were then transferred into a nitrocellulose membrane (Whatman, Dassel, Germany), making a “sandwich” composed of: foam pads, paper, gel, nitrocellulose membrane,

paper and foam pads. The Cassette was introduced into the Blotting tank Module (Bio-Rad; Hercules, CA, USA) and it was filled with 4ºc transfer buffer, composed of 25 mM Tris-HCl, 192 mM glycine, 10% methanol. The transfer was performed on ice to avoid possible overheating for 70 min at 100V.

After the last phase was completed, the membranes were stained with Ponceau-S red in order to check whether the transfer was achieved. Next, the membranes were washed 3 times, 10 min each, with TBS-T composed of 75 mM NaCl, 1.5 mM KCl, 12.4 mM Tris-HCl, and 0.1% Tween-20, pH 7.4 and then blocked for 1 hour at 20-25ºc with Western Blot blocking solution TBS-T with 5% dry nonfat milk in agitation at 300rpm.

Antibodies

The primary antibody was diluted in 5% (w/v) bovine serum albumin (BSA) and incubated overnight with the membranes inside a 50 ml falcon in a roller of agitation at 4ºc. The following list (Table 9) contains the primary antibodies that were used during the experiment:

Table 9: Antibodies, manufacture, dilution and molecular weight used in the experiment.

Primary antibody	Manufacture	Used dilution	Molecular weight
Mouse anti-synaptophysin (SYP) (S5768)	Sigma, USA	1:2500	37 kDa
Mouse anti-tyrosine hydroxylase (TH) (T2928)	Sigma, USA	1:1000	60 kDa
Mouse anti-tryptophan hydroxylase (TPH) (T-06781)	Sigma, USA	1:500	55 kDa
Rabbit anti-Glutamate receptor 1 (AMPA1) (AB1504)	Merck, Germany	1:1000	106 kDa
Mouse anti-Glutamate receptor 2 (AMPA2) (MAB397)	Merck, Germany	1:1000	102 kDa
Mouse anti-NMDAR1 (556308)	BD Biosciences	1:500	120 kDa

Rabbit anti-NMDAR2A (AB1555)	Merck, Germany	1:1000	180 kDa
Mouse anti-GAPDH (G8795)	Sigma, USA	1:10000	37 kDa
Mouse anti-β-Tubulin (556321)	BD Biosciences	1:2000	50 kDa

Anti-GAPDH and anti-β-Tubulin were used as a loading control to normalize the levels of protein detected and to ensure the reliability of the results when comparing expression of a protein. The expression levels of those proteins should not vary between the different samples.

After the primary antibody incubation, three washes of 0.1% TBS-T for 10 min each were performed and the secondary antibody was added for 1 hour at room temperature in Western Blot blocking solution (TBS-T with 5% nonfat dry milk), at 300rpm agitation. The secondary antibodies used were: anti-mouse-HRP 1:2000 (Dako Denmark, Glostrup, Denmark) or anti-rabbit-IgG (H+L) 1:3000 (Pierce, ThermoFisher Scientific, USA). Three more washes with TBS-T were performed for 10 min each.

Image processing and quantification

Blots were developed using a chemoluminiscent mix 1:1 ECL1 (0.5 M luminol, 79.2 mM p-coumaric acid, 1 M Tris-HCl in DMSO; pH 8.5) and ECL2 (8.8 M hydrogen peroxide, 1 M Tris-HCl in DMSO; pH 8.5), and approximately 1 ml of total mix was needed for each membrane and antibody. The apparent molecular weight of proteins was determined by calibrating the blots with pre-stained molecular weight markers, (Precision Plus Protein Dual Color Standards; Bio-Rad, USA) added in one of the wells during the electrophoresis phase. The image processing was carried out using ChemiDoc XRS+ System (Bio-Rad Laboratories). The parameters of time exposition changed between each antibody, but the accumulation signal mode was chosen and the membranes were exposed for a maximum time of 8 min. Chemiluminescence signals of the obtained bands were all within the linear range of the imaging system and were not saturated. Densitometry and quantitation was carried out using ChemiDoc MP Imaging System, Image Lab program (Bio-Rad) and Microsoft Excel was used to determine the levels of proteins. The young group of subjects was used as a control group, and the total level of proteins is expressed as a percentage.

4.3 High Performance Liquid Chromatography

Brain sample homogenization

Following the same procedure described in the sample acquisition paragraph (section 3.4.1), subjects were decapitated and the following brain regions were dissected: striatum, hypothalamus, HPC, frontal and occipital cortices. Right and left regions from striatum, HPC and FC were separated into two identical parts and processed differently: one for the HPLC analysis and the other for Western Blot analysis. All the regions were stored in individual 1 ml Eppendorfs and weighted. The HT and the occipital cortex were only analyzed via HPLC. The tissue dissection was performed under ice-cold conditions. Finally, the brain sections were stored at -80ºC.

Brain samples were homogenized in buffer (perchloric acid 60% w/w 0,25M, sodium metabisulphite 100 µM, EDTA Na_2 $2H_2O$ 250 µM) in a 9/1 ratio (p/v; ml/mg). A polytron homogenizer was used in order to break down the animal tissue for no more than 10s. Then, the homogenated samples were centrifuged (10 min at 4000 rpm, 4ºC) and the supernatant filtered. Samples were maintained at -80ºC during the whole procedure.

Analysis of samples

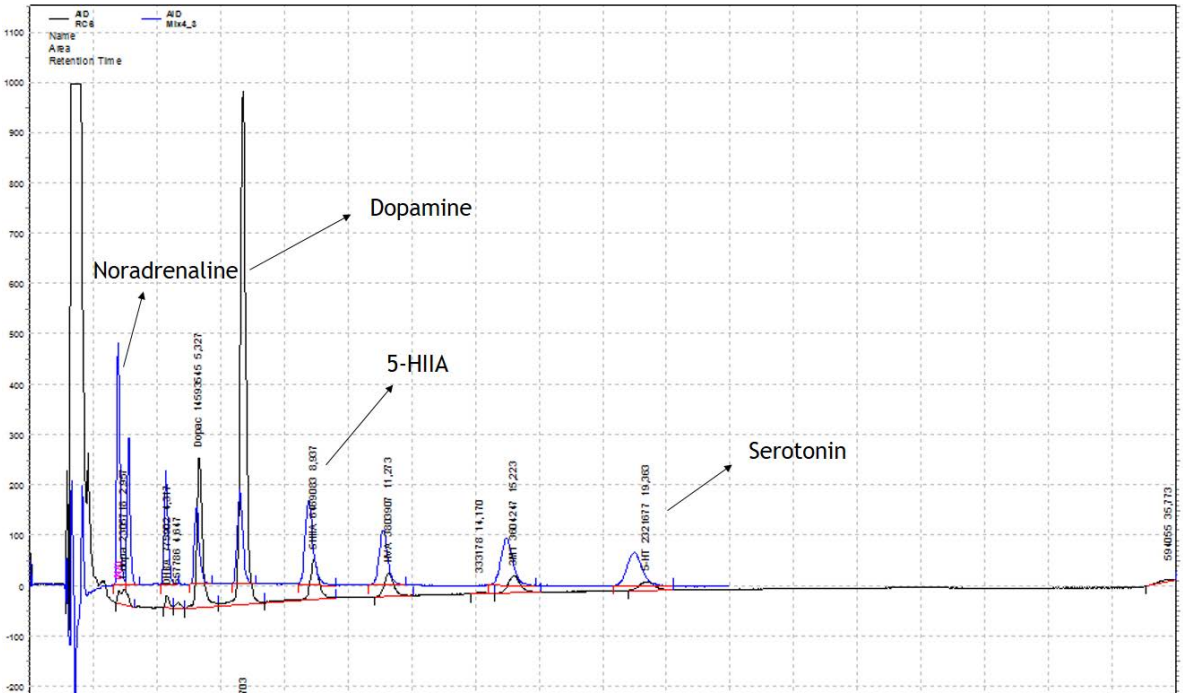


Figure 17: Image of the monoamines levels in mix 4_3 (blue) and sample RC_6 (black) overlapping (pg/mL).

Aliquots of 50 µL from the purified supernatants were subjected to HPLC analysis in an inverse phase column (Cromolith Performance 4.6 mm internal diameter x 10 cm longitude) coupled with a pre-column (4.6 mm x 5 cm). The mobile phase consisted of a buffer with citric acid 0.1M, EDTA 0.05M, SOS 1.2m, acetonitrile 10% (v/v) with a pH of 2.75, adjusted with tetraethyl ammonium. The elution was pumped at a flow rate of 0.8ML/min. The HPLC equipment

(LaChrom Elite) was coupled to an ESA Coulochem 5100A electrochemical detector with an ESA 5011A analytic cell with double electrode. The contents of precursor of amino acids 5-HTP and DOPA, monoamines 5-HT, DA, and NA, metabolites 5-HIAA and homovanillic acid (HVA) were detected electrochemically using a cell with two electrodes (detection potential for the electrodes 1 and 2 was fixed on 70.05 and +0.4 V each). The current produced was monitored by an interface connected to a monitor PC. A calibration mix of the different components revealed the best previously known concentration, which was used to perform a quantification curve each time the samples were analyzed. Each concentration level had all the monoamines and their metabolites, the different levels were created through a series of varying dilutions of the stock standard by transferring known volume of the standard and adding solvent. The series included a total of seven concentrations for calibrating the HPLC (9.375, 18.75, 37.5, 75, 150, 300 and 600 µg/mL). With this, a calibration curve (r^2= 0.999) was obtained and used to identify and quantify the concentrations of the different monoamines and their metabolites. EZChrom Elite Software (Figure 17) was used to determine concentrations, which are expressed in ng/g tissue (calculated from pg/mL: results from the HPLC were multiplied x 9 because originally the samples were homogenate in a proportion of x 9 each sample weight).

4.4 Blood plasma analysis

Blood collection

As described in the sample acquisition (Section 3.4.1), at the decapitation of the animals a blood sample was collected and preserved with Heparin (Sodium Heparin, 5000USP/mL; Chiesi España SA, Spain) to avoid coagulation. Then, the blood sample was rapidly centrifuged for 15 min at 4ºC at 3500 rpm. Once the sample was centrifuged, the supernatant (plasma) was separated from the blood and frozen at -20ºC for a better preservation.

Plasma analysis

The plasma analysis was carried out externally, by the *Servei de Bioquímica, Clinica Veternitaria, Universitat Autònoma de Barcelona*. The parameters analyzed were: Corticosterone, Insulin, Leptin, IGF-1, Cholesterol, Glucose, Total Proteins, Triglycerides, Albumin, Low-density lipoprotein (LDL), High-density lipoprotein (HDL) and ALP.

The following methodology was performed to determine each parameter level:

Corticosterone levels were measured using a competitive ELISA method, EMS Reader MF V.2.9-0, with a Corticosterone EIA reactive (Immunodiagnostic Systems Ltd, IDS Ltd; Boldon, United Kingdom). Insulin, Leptin and IGF-1 levels were calculated using a Sandwich ELISA method, EMS

Reader MF V.2.9-0, with the following reactive for each protein, Mercodia Rat Insulin ELISA from Mercodia AB, Sweden, Quantikine® ELISA Mouse/Rat leptin, of R&D Systems, Inc.USA, Quantikine® ELISA Mouse/Rat IGF-1, de R&D Systems, Inc.USA.

Cholesterol analysis was performed using the Enzyme method CHE/POD1; glucose levels were detected with the Hexokinase method; total protein was calculated by the application of the Biuret method; triglycerides quantification was carried out with the Glycerol-3-phosphate oxidase method, albumin by the Bromocresol green method; the determination of calcium levels were performed by the Arzenazo III method; LDL-c with the selective protection method; HDL with the immunoinhibition method; and the ALP levels were examined by the Substrate 4-nitrophenyl phosphate with AMP tampon methodology. All of the tests described above were carried out using the analyzer Olympus AU400 (Germany), as well as the reactive OSR (Olympus System Reagent, Beckman Coulter®, Ireland).

5. Statistical analysis

Behavioral and biochemical data were analyzed using the Statistical Package for the Social Sciences (SPSS) v22 software (Chicago, IL, USA) and plotted as mean ± SEM. Detection of outliers was performed with boxplot analysis and, when necessary, removed from the analysis. A test of normality and homoscedasticity of variances; Levene's test, was applied to each data set before the one-way analysis of variance (ANOVA) analysis. If Levene's test was significant and the assumption of homogeneity of variances was violated, a Welch's ANOVA test was carried out. Comparisons among the three experimental groups (*Ad Libitum,* CR and Young) in all the variables were completed with standard one-way ANOVA, except in the case of the analysis of the results of MWM, in which ANOVA for repeated measures was carried out for the 5-day acquisition phase. In addition, in the test phase of MWM and Object recognition in the Y maze, a one-sample t-test against a constant (25 in MWM and 50 and 33 in Object recognition) was used for each group to determine whether the percentage of time in the target quadrant or in the target arm was different from the chance level (25%, 50% or 33%). Moreover, Spearman Rank correlations were conducted in order to examine the relation between different variables. Positive coefficients indicate a direct relationship; as one variable increases, so does the other variable. Negative correlations coefficients indicate an indirect relationship; as one variable increases, the other variable decreases. Results were considered significantly different at $p < 0.05$.

The variables analyzed for each behavioral task were:

OF: permanency in each zone, mean speed and number of entrances to each zone.

EPM: total number of entrances to each arm, two different anxiety ratios (total time in open arm/total time of the task and number of open arm entries/ total number of entries).

MWM: During the acquisition phase: the latency to find the platform, swim speed, time at platform quadrant, total length (total distance swum) and *thigmotaxis* (percentage of time spent near the walls). In the test phase: the percentage of time spent in the target quadrant (time spent in the quadrant where the platform was versus the time spent in the other quadrants), and in the target annulus (the area surrounding the location of the platform in the previous phase), swim speed, total length travelled, *thigmotaxis*, and number of target crossing. In the reversal phase: the mean latency to find the new location of the platform, swim speed, length and *thigmotaxis*.

ODT: The latencies to find the rewarded sponge and start eating the chocolate reinforcement and the number of two different types of errors (commission and omission).

Olfactory perception test: The latency to find the buried cookie and beginning to eat it.

Object recognition in Y maze: The first choice (first visited arm), the time exploring each arm and the time exploring the novel object.

The biochemical data provided was also analyzed via ANOVA and the investigated variables were:

HPLC: Concentration of neurotransmitters and metabolites.

WB: The variation of the levels of each intensity band expressed protein compared to the control young group (100%).

Plasma analysis: Mean plasma levels of each determinant.

After such an extensive variable analysis, an ANOVA analysis, followed by Post Hoc contrasts (multiple comparisons were performed with the Bonferroni correction), assessed the effects of Group (*Ad Libitum*, CR or Young) on all the behavioral tasks, except for the previously mentioned cases (variables from the acquisition phase of the MWM).

Correlations: In order to study a possible relation between the biochemical variables and the behavioral results, different Spearman's correlations were performed (section results: Correlations). A subgroup of behavioral variables was chosen for being the best indicators of

learning and memory retention. Concretely, learning was evaluated by the latency to find the platform, or the time in the target quadrant in the last acquisition session of the MWM, or the mean latency to find the reinforcement in the ODT. Long-term memory was demonstrated by the time at the quadrant platform or target annulus during the test phase in the MWM and the latency to find the reinforcement in the test session in the ODT. Regarding the biochemical variables, the ones which were most related to age-related cognitive deterioration were chosen. Specifically, we focused on the glutamatergic and monoaminergic systems in the HPC and FC, as well as peripheral hormones parameters. Out of all of the correlations carried out, only those that revealed the most interesting results in regard to the objectives proposed for our research will be described.

V. RESULTS

V. RESULTS

1. Behavioral data

1.1 Open Field

Table 10 shows the percentage of permanency (± SEM) in the three zones during OF performance for the three groups of rats. ANOVA confirmed no statistical differences between groups in time of permanency in the different zones of the OF (Zone 1: $F_{(2, 30)}$= 0.214, p = 0.809; Zone 2: $F_{(2, 30)}$= 0.306, p = 0.739; Zone 3: $F_{(2, 30)}$= 0.919, p = 0.410), indicating that neither aging nor CR had an effect on locomotor activity of the rats.

Table 10: Percentage (%) of permanency in each zone during OF performance (mean ± SEM).

	Zone 1 (exterior)	Zone 2 (medium)	Zone 3 (interior)
Ad Libitum	89.945 ± 3.553	9.581 ± 3.762	0.473 ± 2.282
CR	89.331 ± 3.486	10.207 ± 3.622	0.46 ± 2.022
Young	92.08 ± 0.144	7.085 ± 0.153	0.825 ± 0.296

Figure 18 shows the number of entries (± SEM) to each zone of the OF, during the 15 minutes of free exploration, being zone 1 the innermost, zone 2 the medium area and the zone 3 the outermost, for the three groups. ANOVA showed no differences in the number of entries to each zone of the OF between the three groups (Zone 1 $F_{(2, 30)}$= 0.724, p = 0.493, Zone 2 $F_{(2, 30)}$ = 0.823, p = 0.449 and Zone 3 $F_{(2, 30)}$= 0.962, p = 0.394), demonstrating there was no effects of age or CR on this variable.

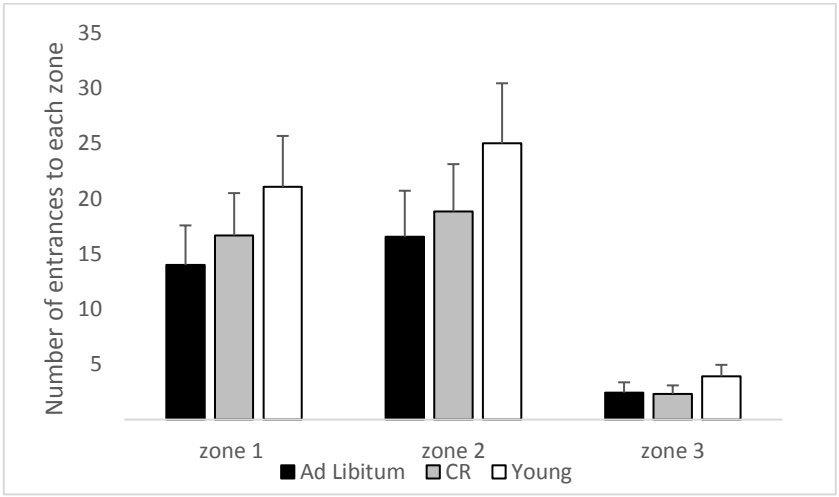


Figure 18: Number of entrances (± SEM) to each zone during OF performance for each group.

Regarding the variable of speed, Figure 19 represents the average speed of ambulation (± SEM) during 15 minutes of free exploration in the OF for the three groups of animals. ANOVA showed

statistically significant differences between groups in the mean speed ($F_{(2,30)}$= 5.701, p = 0.008). A Post Hoc analysis indicated significant differences between Young and CR groups (p = 0.010) and a tendency to significance between Young and *Ad Libitum* groups (p = 0.057).

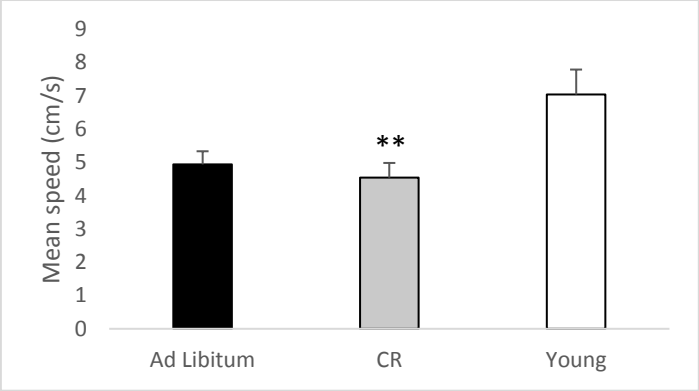


*Figure 19: Mean speed (± SEM) during OF performance for each group. **p< 0.01 between CR and Young group.*

This demonstrates an effect of age upon this variable, as both aged groups of animals were slower than the Young animals.

1.2 Elevated Plus Maze

Figure 20 represents the number of entries to each arm during the 5 minutes of free exploration of the EPM for the three groups of animals. ANOVA showed no differences between groups in the total number of entries into the open arm ($F_{(2,30)}$= 0.210, p = 0.811), neither in the number of entries into the end of the open arms ($F_{(2,30)}$= 0.318, p = 0.730). However, significant differences between groups in the number of entries to the closed arms ($F_{(2,30)}$= 6.810, p = 0.004) were detected. A Post Hoc analysis presented differences between the CR and Young groups (p = 0.03), showing that CR aged animals made less number of entries into the closed arms.

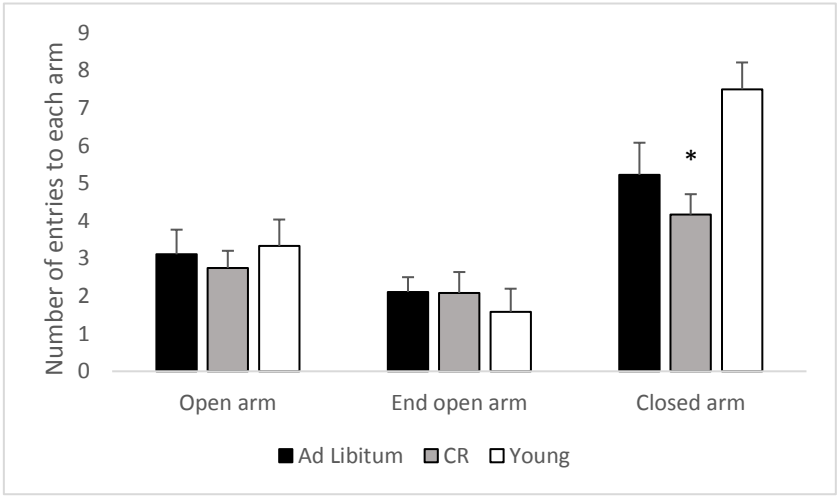


*Figure 20: Number of entries (± SEM) to each arm in EPM performance for each group. *p < 0.05 between the CR and Young group in the closed arm.*

Figure 21 depicts the percentage of permanency in open arm time (± SEM) in relation to the total time of the test (300s) for the three groups of animals. This ratio is used as an anxiety variable, since more time spent in the open arm indicates more anxiety, as previously explained (Methodology 2.2). The results via ANOVA showed no differences between groups ($F_{(2,30)}$= 1.427, p = 0.256), indicating no effects of neither age nor CR effect upon this variable.

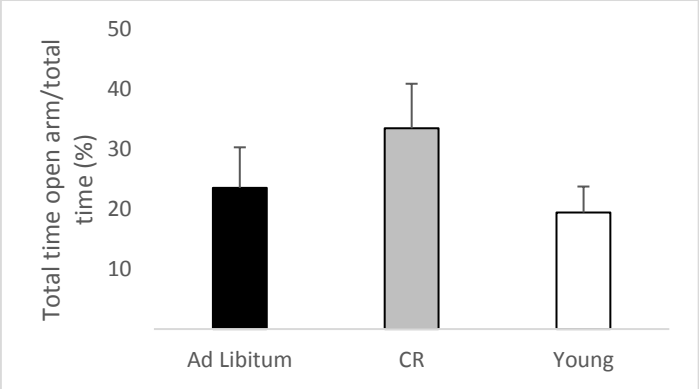


Figure 21: Total time in open arm/total time (± SEM) expressed as a percentage, during the EPM performance for each group.

Figure 22 shows an alternative way to analyze the level of anxiety, which involves calculating a percentage (± SEM) of the ratio of total number of entries to the open arm in relation to the total entries to both open and closed arms. Statistical analysis of this variable via ANOVA showed no differences between groups ($F_{(2, 30)}$ = 1.871, p = 0.172).

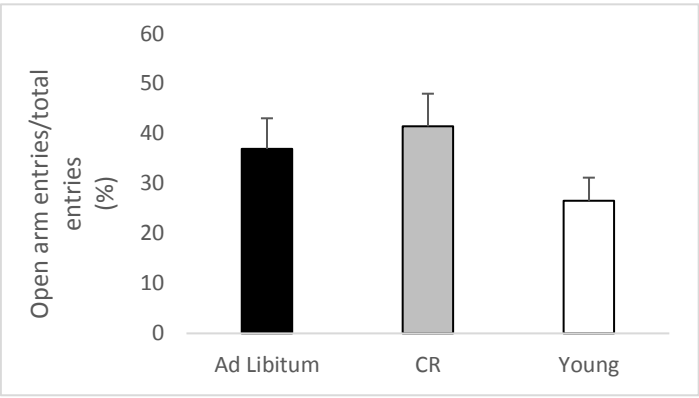


Figure 22: Open arm entries/total entries (± SEM) expressed as a percentage, during the EPM performance for each group.

Partial discussion for Open Field and Elevated Plus Maze

The OF and EPM are usually used to study anxiety-related behavior (Carola et al., 2002) and locomotor activity in rodents (Kuhla et al., 2013). Typical exploratory behavior in the OF test consists in the exploration of the outer zone (*thigmotaxis*), while a more extended exploration in the central zone is indicative of less anxiety. Therefore, number of entries into the central and middle zones were used as an anxiety-related measure (Levay et al., 2007). Our results are similar to those observed in a study in mice (Kuhla et al., 2013), in which animals received a 40% CR diet for 74 weeks. They found that CR did not have any effects on the age-related loss of spontaneous motor activity in the OF, as both aged groups were slower. However, our results are in contrast to another study (Geng et al., 2007) which found that the CR group had a higher

spontaneous locomotor activity compared to the *Ad Libitum* group of old rats. It is important to consider the CR applied consisted in a 40% reduction of food intake, what is higher than the 25-30% applied by the present study.

Moreover, the EPM is used to examine anxiety through the analysis of the time the animals spend in the open arms; the more time spent in the open arms, the less anxiety. No differences between groups were found in any of the anxiety related variables. Furthermore, the number of closed arm entries have been found to provide a reliable measure of locomotor activity (Levay et al., 2007). In the present experiment, Young animals visited the closed arm more times than the CR group, which can be interpreted as higher locomotion. Contrary to our results, findings from another study (Jahng et al., 2007) showed that CR treatment, applied to young Sprague-Dawley rats, increased the anxiety levels of the animals, since CR-treated young rats spent more time in the closed arms and less time in the open arms when compared to control animals. However, the animals were subjected to a 50% CR treatment, which is classified as semi-starvation.

Given our findings in the EPM, together with results in mean speed in the OF, we can conclude that the CR group showed decreased locomotor activity compared to the Young group of animals, as they did not differ from *Ad Libitum* aged group. Thus, it could be interpreted that aging slowed down locomotor activity and that CR did not ameliorate this effect. Moreover, there is another variable that we need to consider to help interpret our results, which is the time of the day in which the food was delivered to the rats. In the present study, the behavioral part of the experiment took place one to two hours after supplying the food to the CR animals, which were fed only once a day with the total amount of food. Such procedure might have influenced the irritability and level of activity in the CR animals. In fact, a previous study (Smith & Metz, 2005) demonstrated that *Ad Libitum* rats showed more movement accuracy than CR animals. This could help explain the slight affectation of the CR group in the control tasks. However, the locomotor activity in other tasks was not impaired in the CR group, as presented in the following section (Figure 24). To conclude, results obtained for OF and EPM indicate that all the animals had a similar anxiety-like behavior and CR only slightly affected the locomotor activity of the CR rats, an effect that can be attributed to aging.

1.3 Morris Water Maze

Acquisition phase

Figure 23 depicts the mean latency (in seconds) to find the hidden platform (± SEM) in the MWM during five consecutive days of acquisition, four trials each day, for the three experimental groups. The results revealed that all animals progressively reduced the latency to locate the platform during acquisition. ANOVA with repeated measures showed significant differences between sessions ($F_{(4,172)}$= 19.137, p < 0.001), between groups ($F_{(2,43)}$= 14.512, p = 0.001) and interaction group X session ($F_{(8,172)}$= 2.428, p = 0.016), indicating that the evolution of the performance across the five sessions was significantly different between groups. The Post Hoc analysis confirmed significant differences between the groups *Ad Libitum* and Young on day 1 (p = 0.016), day 3 (p = 0.001), day 4 (p = 0.002) and day 5 (p < 0.001). There were also differences between CR and Young groups on day 2 (p = 0.016), day 3 (p = 0.001), day 4 (p = 0.006) and day 5 (p < 0.001). In general, these findings demonstrate an age effect across the phase, indicating that both aged groups presented a worsened learning process. However, they showed to have learned the task, since the latency to find the platform progressively decreased.

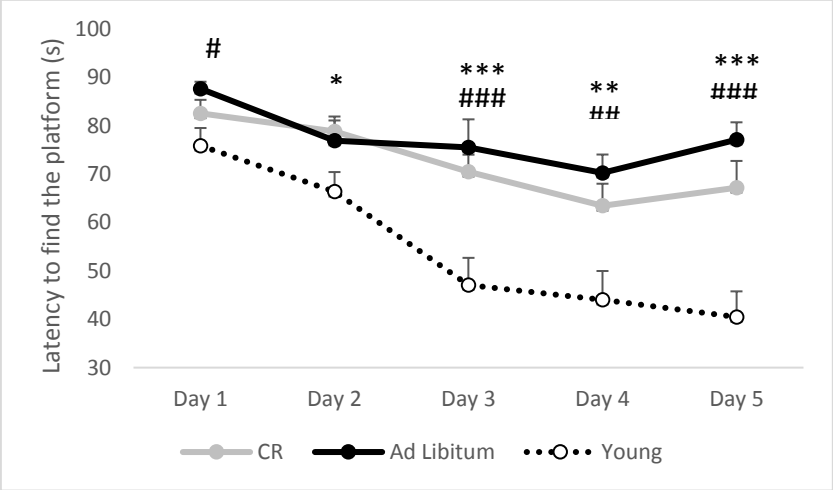


*Figure 23: Latency to find the hidden platform (± SEM) during the acquisition in the MWM for each group. Significant differences between CR and Young groups *p<0.05, **p<0.01, ***p<0.001 and between Ad Libitum and Young groups #p<0.05, ##p<0.01, ###p<0.001.*

Figure 24 depicts the control variable for swim speed (the mean velocity) in cm/s (± SEM) during all the acquisition sessions for the three groups of rats. ANOVA with repeated measures shows statistically significant group x session interaction ($F_{(8,172)}$= 4.808, p = 0.001) and differences between sessions ($F_{(4,172)}$= 8.041, p < 0.001) but no between groups ($F_{(2,43)}$= 1.276, p = 0.290). The differences were detected on day 1, between CR and Young groups (p = 0.003) and between *Ad Libitum* and Young groups (p = 0.008), and on day 2 between *Ad Libitum* and Young groups (p = 0.003). Because these were the initial sessions, the novelty of the task may have affected

the speed, but later sessions show that all the groups had a normal execution and similar motor activity in the pool.

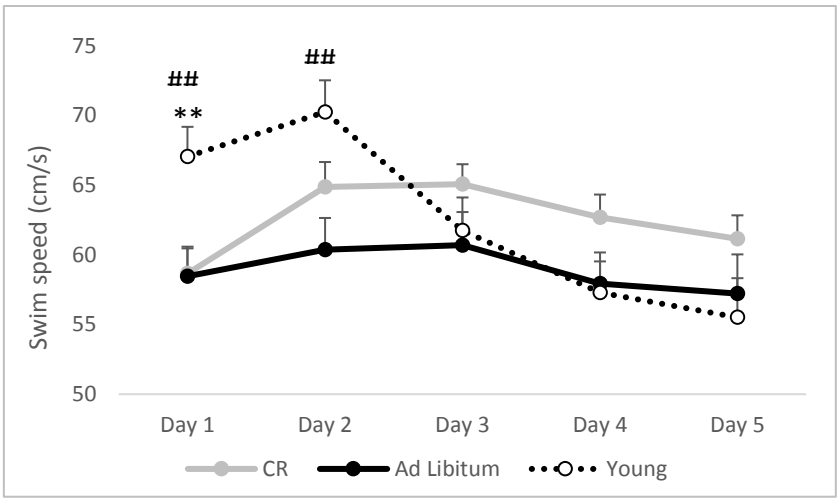


*Figure 24: Swim speed (± SEM) during the five days of acquisition in MWM for each group. Significant differences between CR and young groups **p< 0.01 and Ad Libitum and young groups ##p< 0.01.*

Figure 25 shows the time in percentage (± SEM) at the quadrant where the platform was in each acquisition day for each group of rats. ANOVA with repeated measures showed no interaction between groups and sessions ($F_{(8,172)}$= 1.587, p = 0.132), but significant differences between sessions ($F_{(4,172)}$= 14.58, p = 0.001) and between groups ($F_{(2,43)}$= 23.913, p = 0.001), which indicates that all the animals performed in a similar way during the learning phase. Although they all remembered the position of the platform, the Young animals spent more time in the target quadrant than both aged groups.

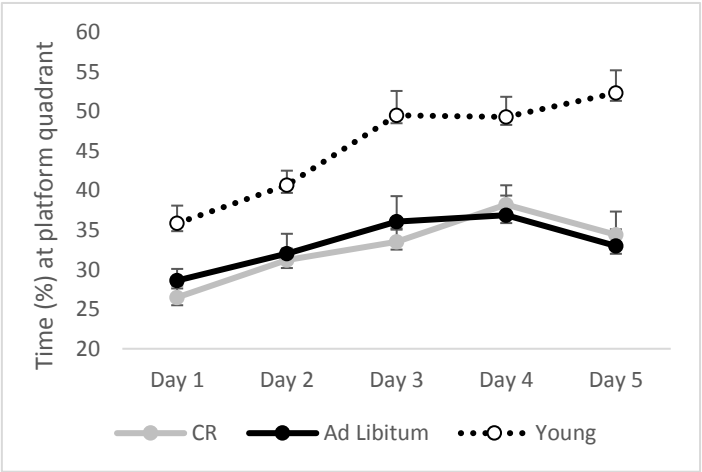


Figure 25: Time at the platform quadrant in percentage (± SEM) during the five days of acquisition in the MWM for each group.

Figure 26 shows the total length in cm (± SEM) travelled during the five sessions of acquisition for the three groups of animals. ANOVA with repeated measures showed interaction group session ($F_{(8,172)}$ =3.616, p = 0.001) and significant differences between groups ($F_{(2,43)}$= 6.023, p = 0.005) and sessions ($F_{(4,172)}$= 15.218, p = 0.001). The Post Hoc analysis confirmed significant differences between the Young and the *Ad Libitum* groups (p = 0.012) on day 3 (p = 0.010), day

4 (p = 0.019) and day 5 (p = 0.001), as well as between the Young and CR groups (p = 0.017) on day 3 (p = 0.004), day 4 (p = 0.017) and day 5 (p = 0.001). This demonstrates an age effect of this variable, as the young group of animals travelled shorter distances to find the scape platform.

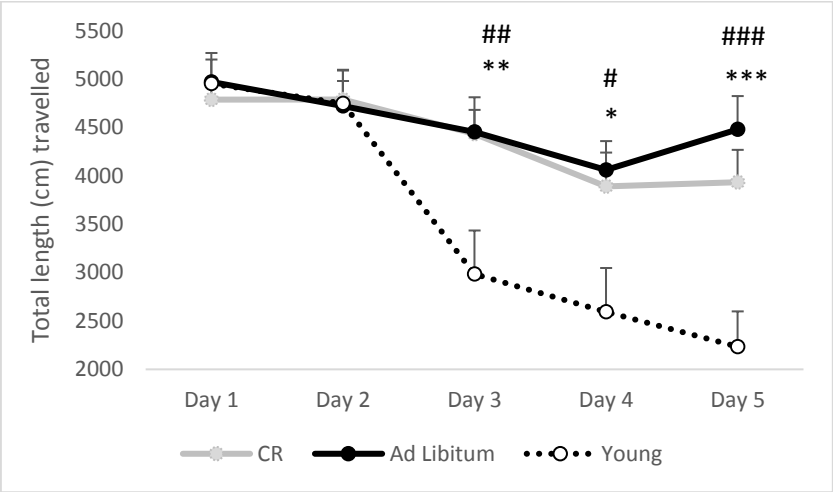


*Figure 26: Total length travelled (± SEM) during the acquisition phase in the MWM for each group. Significant differences between CR and Young groups *p<0.05, **p<0.01, ***p<0.001 and between Ad Libitum and Young groups #p<0.05, ##p<0.01, ###p<0.001.*

Figure 27 depicts the control variable *thigmotaxis*, an index of anxiety, defined as the total time that the animal swam or stayed near the walls (± SEM) for the three groups of animals. ANOVA with repeated measures showed no interaction group per session ($F_{(6.48,139.35)} = 0.621$, p = 0.725), neither statistically significant differences between groups ($F_{(2,43)} = 2.663$, p = 0.081), but between sessions ($F_{(3.24,139.35)} = 51.559$, p = 0.001) indicating that all groups of animals presented a similar level of anxiety during the acquisition phase.

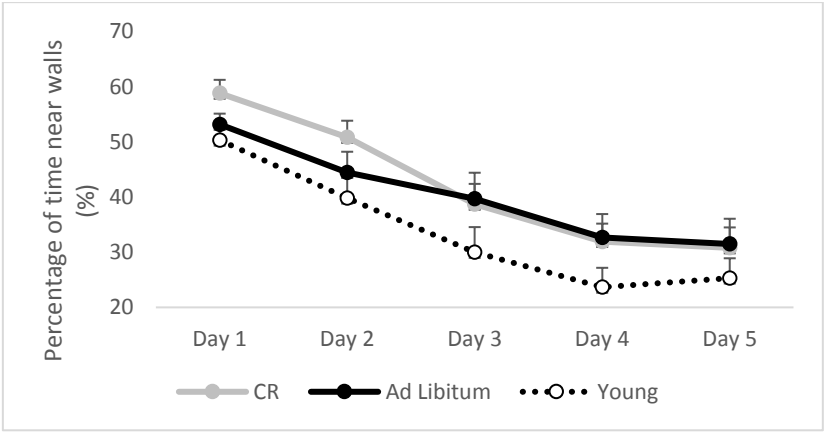


Figure 27: Total time spent near the walls (± SEM) in percentage during the MWM acquisition for each group.

Test Phase

Figure 28 shows the time spent at the target quadrant (± SEM) during the 72h-probe memory test, in which the platform has been removed from the pool. The dotted line crossing the Figure indicates the fix value of 25%, namely the total time that an animal remained in one quadrant, and the line above bars represents the statistical difference between groups. ANOVA confirmed statistically significant differences between groups ($F_{(2,43)}$= 4.985, p = 0.011) and a Post Hoc analysis indicated differences between Young and Old *Ad Libitum* groups (p = 0.009). T test with a fix value of 25 (random) showed no differences from chance level for the *Ad Libitum* group ($t_{(10)}$= 0.351, p = 0.733), but there was a significant difference for the CR group ($t_{(18)}$= 4.824, p < 0.001) and for the Young group ($t_{(15)}$= 3.636, p = 0.002). These results indicate that the Young and the CR groups remembered the locations of the platform, since the time they spent in the target quadrant exceeded chance level. In contrast, *Ad Libitum* animals performed worse than the CR and Young animals and did not remembered the platform location, since the time at the platform quadrant did not differ from chance level. In conclusion, CR improved MWM memory in old rats.

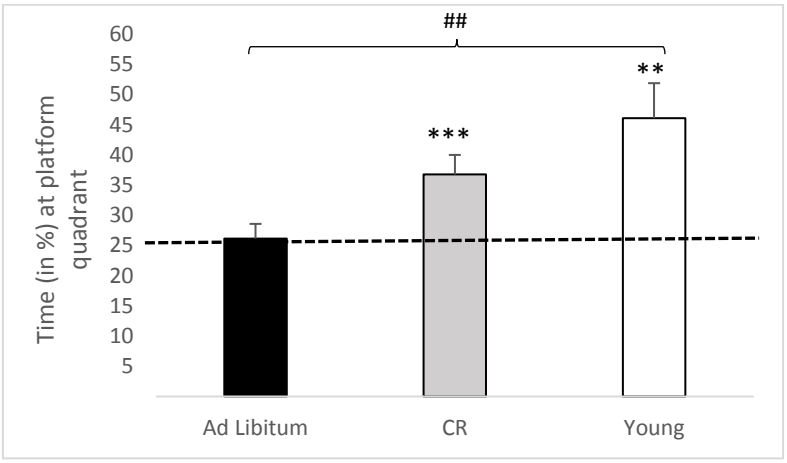


*Figure 28: Time at platform quadrant (± SEM) during the test phase of the MWM for each group. ANOVA is indicated with ##p <0.01, statistical difference between Ad Libitum and Young group. T-test with a fix value of 25 is indicated with **p < 0.01 for the Young group and ***p < 0.001 for the CR group.*

Figure 29 shows the meantime (in percentage) spent in the target annulus during the test. ANOVA showed differences between groups ($F_{(2,43)}$= 4.897, p= 0.012), and a Post Hoc analysis detected differences between *Ad Libitum* and Young groups (p= 0.011). This result agrees with the time at the platform quadrant, confirming that CR aged animals had a better memory of the task than the *Ad Libitum* old rats.

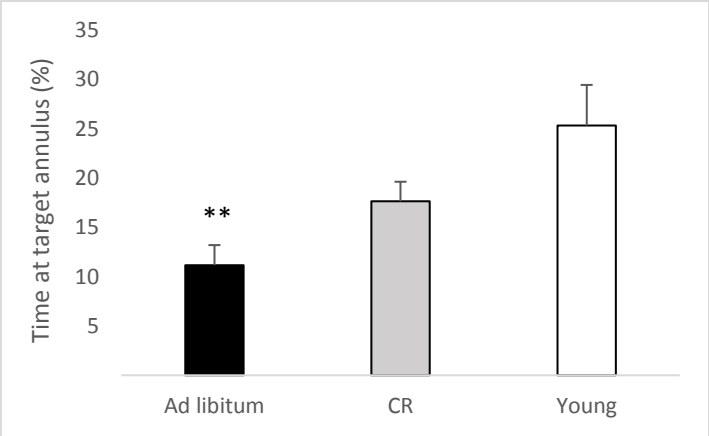


*Figure 29: Mean time in percentage (± SEM) at target annulus during the test of the MWM for each group. **p < 0.01 between Ad Libitum and Young groups.*

Figure 30 shows the mean swim speed in cm/s (± SEM) of the three groups of animals during MWM test. ANOVA showed no differences between groups ($F_{(2,43)}$= 1,416, p = 0,254), suggesting there were no locomotion impairments in the old animals.

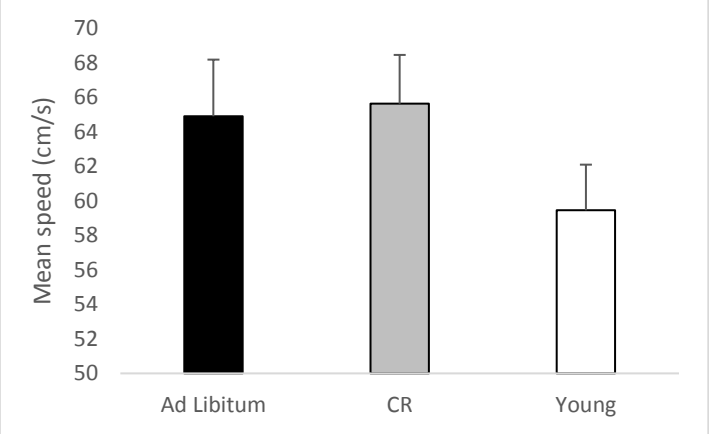


Figure 30: Mean speed (± SEM) during the MWM test for each group.

Figure 31, shows the mean length travelled, in centimeters (± SEM), during the 60s of the MWM test for the three groups of animals. ANOVA showed no differences between groups ($F_{(2,43)}$ = 1.273, p = 0.290), indicating that all animals had a similar strategy to search for the platform.

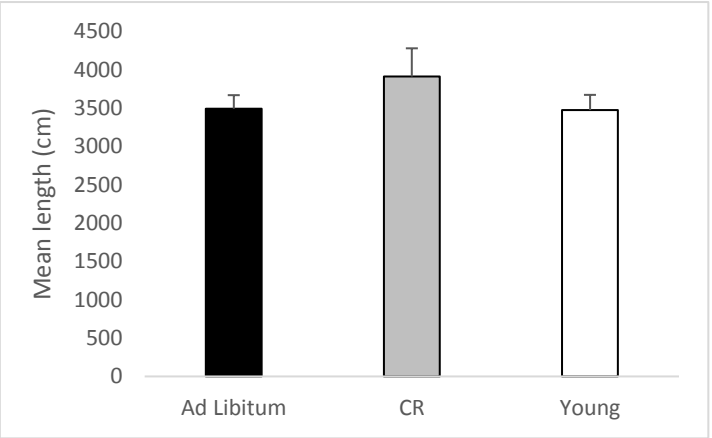


Figure 31: Mean length travelled in cm (± SEM) during the MWM test for each group.

Figure 32 depicts the time the animals spent near the walls, also called *thigmotaxis*, (± SEM), during the test of the MWM. ANOVA showed no differences between groups ($F_{(2,43)}$ = 0.548, p = 0.582), therefore, all animals showed similar levels of anxiety in the execution of the probe test.

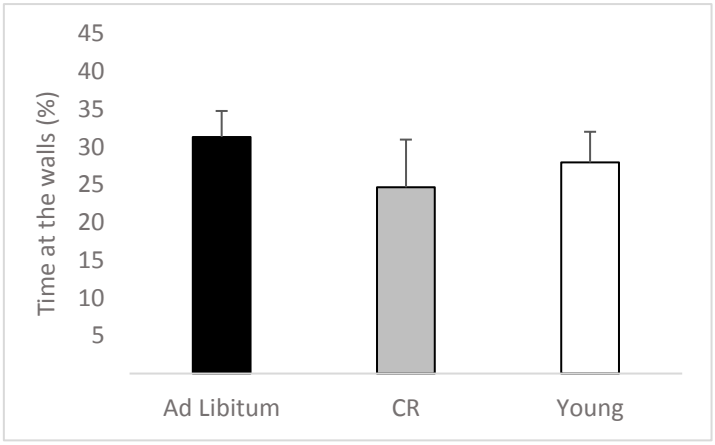


Figure 32: Mean time spent in the walls (± SEM), "thigmotaxis", during the test phase of the MWM for each group.

Figure 33 shows the number of times that the animals crossed the precise location of the hidden platform (± SEM) during the test. ANOVA showed no differences between groups ($F_{(2,43)}$ = 0,010, p= 0,990), demonstrating there were no effects of neither age nor CR on this variable, since all animals had a similar performance.

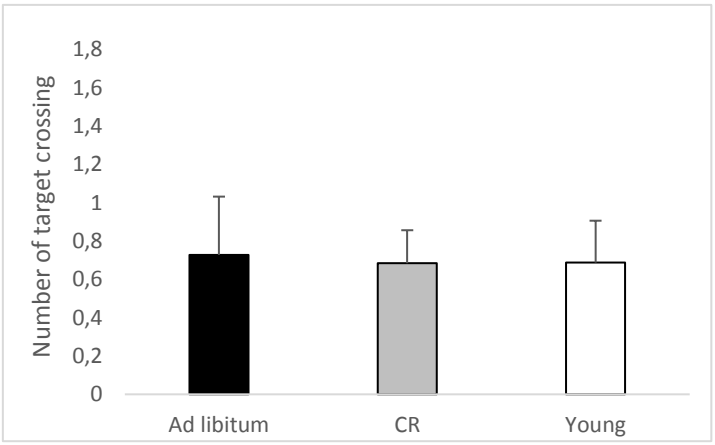


Figure 33: Number of target crossings (± SEM) where the platform was situated during the test phase of the MWM for each group.

Reversal Phase

The different variables analyzed during the three trials of reversal learning performed after the test (Methodology 2.3) were: mean latency to find the platform, mean swim speed, mean length travelled and *thigmotaxis* (Table 11). ANOVA indicated that there were no differences between groups in the latency (s) to find the new location of the platform ($F_{(2,43)}$ = 1.630, p = 0.208), mean swim speed (cm/s) ($F_{(2,43)}$ = 0.967, p = 0.388), the mean length (in cm) travelled to find the new location of the platform ($F_{(2,43)}$ = 0.835, p = 0.441), or in the *thigmotaxis* variable ($F_{(2,43)}$ = 0.598, p = 0.554). These results indicate that all groups learned the new location of the platform.

Table 11: Different variables analyzed during the reversal in the MWM (Data show means of three sessions ± SEM).

	Mean latency to find the platform (s)	Mean swim speed (cm/s)	Mean length travelled (cm)	Time at the walls (*Thigmotaxis*) (%)
Ad Libitum	65.194 ± 4.169	52.774 ± 2.531	3496.287 ± 434.911	28.453 ± 5.6791
CR	64.980 ± 6.688	56.291 ± 2.052	3655.571 ± 297.103	24.518 ± 3.148
Young	53.843 ± 5.181	57.182 ± 1.906	3090.678 ± 305.922	22.119 ± 3.214

Partial discussion for the Morris Water Maze

The MWM is a spatial learning test in which animals rely on distal cues to navigate from a start point located in the perimeter of a swimming pool, in order to find a submerged escape platform. Spatial learning is assessed across repeated trials and memory is determined by preference for the platform area when the platform is absent (Vorhees & Williams, 2006). Results of our experiment revealed that all groups gradually reduce their latencies to locate the platform, which is an indicator of spatial learning (Gallagher et al., 1993). Our results demonstrated an age-related cognitive decline across the learning phase (Adams et al., 2008) as previous literature predicted (Geng et al., 2007; Kuhla et al., 2013; Nakamura & Ohno, 1995; Stewart et al., 1989; Wang et al., 2007). Furthermore, young animals displayed a better ability to learn the location of the platform in a short period of time (Gage et al., 1984; Carter et al., 2009). A restriction in the caloric consumption proved to be beneficial for the aged animals, since CR group performed better than the old *Ad Libitum* rats. However, the improved performance was only observed in some, but not all of the variables analyzed, which supports what previous studies have reported (Adams et al., 2008; Gyger et al., 1992; Markowska, 1999). However our results did not agree with other reports that found a positive effect of CR on MWM acquisition in old rats (Carter et al., 2009; Stewart et al., 1989) and mice (Kuhla et al., 2013). Overall, our results were consistent with findings in previous studies. Nevertheless, it is important to know which experimental design was applied in each phase, since there can be many variations, including the number of trials per session and the number of sessions in total. For example, in a previous study (Adams et al., 2008), the acquisition phase was comprised of 5 trials per day for 4 days, while the intertrial time was between 3 to 5 min. Both parameters differ from our procedure (Methodology 3.2.3), which could help explain the differences in outcome.

The spatial memory accuracy was analyzed through the percentage of time spent in the platform quadrant (D'Hooge & De Deyn, 2001), in the test phase of the MWM. It was performed 72 h after the last acquisition session. The results indicated that CR and Young groups of animals correctly learned and/or recalled the platform location. In contrast, *Ad Libitum* did not. These

findings suggest that life-long CR led to small but significant improvements in the performance of the MWM in aged rats, in agreement with previous reports (Stewart et al., 1989) and confirming that CR in old rats can ameliorate age-dependent spatial memory decline, as has been previously reported (Geng et al., 2007; Gyger et al., 1992). Other variables such as mean length travelled and target crossings are similar between groups, revealing that there were no significant differences in the spatial strategy used by the subjects to search the platform during the test phase (Cardoso et al., 2016).

The reversal phase involves the ability to rapidly modify responses in order to adapt to a changing task, as well as the ability to inhibit the initial learning and acquire a new one. The changing actions that occur in the reversal phase are related to a flexible cognitive capacity (Vorhees & Williams, 2006). In addition, reversal trials enhance the detection of spatial or cognitive impairments (Thong-Asa et al., 2012). This phase was performed immediately after the test, and similar execution between groups was found, demonstrating adaptive learning. However, previous studies (Leite-Almeida et al., 2009; Portero-Tresserra et al., 2018) demonstrated that aging impairs the reversal spatial learning. In addition, it has been suggested (Nieves-Martinez et al., 2012) that cognitive rigidity increases with age. Furthermore, a previous study (Gyger et al., 1992) in CR and aging demonstrated that 30% calorically restricted 24-month-old rats adjusted to the new location of the platform, but the *Ad Libitum* aged animals did not. These findings did not support our results, since aging did not impair the cognitive flexibility and CR did not affect the reversal results. Surprisingly, a recent study from our laboratory using a similar MWM protocol (Portero-Tresserra et al., 2018), demonstrated that 24-month-old aged animals performed significantly worse than young controls. However, the old animals also presented an age-dependent increase in thigmotatic behavior during the acquisition, test and reversal phases. This behaviour may have prevented them from adopting an accurate spatial search strategy (Anderson et al., 2014). In contrast, our aged animals did not show higher anxiety levels, as previously demontrated in the OF and the EPM. In addition, all groups presented similar anxiety levels, what lead us to conclude that cognitive decline in aging is marked by considerable variability, with some individuals experiencing significant impairments and others retaining intact functioning (Anderson et al., 2014). Thus, further research into the effects of CR on the reversal phase must be performed in order to clarify present results.

Regarding the control variables in the three phases of the MWM, *thigmotaxis*, a measure that indicate fearfulness (Von Lubitz et al, 1993) was similar between groups, indicating that anxiety did not affect the learning process or memory recall (Nieves-Martinez et al., 2012). On the other

hand, an effect of age on the speed in the first sessions of acquisition was found, which is consistent with previous reports of speed decline in aged rats (Carter et al., 2002; Leite-Almeida et al., 2009; Portero-Tresserra et al., 2018). However, all the animals showed a comparable speed for the remaining learning procedure. A similar outcome has been previously reported in 18-month-old mice compared to young 6-month-old animals (Svensson et al., 2006). Therefore, our results indicated that old rats did not have locomotor or anxiety problems during the performance of the task.

1.4 Olfactory Discrimination Task

Three days of habituation to the food took place prior to the ODT acquisition phase, in order to avoid the risk of *neophobia* to the reinforcement, the chocolate cereals. Table 12 shows the mean time in seconds (± SEM) that animals took to eat all the nine pieces of chocolate cereal scattered all over the cage, for the three groups of animals. ANOVA with repeated measures indicated no interaction group per session ($F_{(4,86)}$= 2.485, p = 0.059) but statistically significant differences between sessions ($F_{(2,86)}$= 33.781, p < 0.001) but not between groups ($F_{(2\ 43)}$ = 2.490, p = 0.095), demonstrating that all animals took a similar amount of time to eat the reinforcement.

Table 12: Mean time (s) (± SEM) the animals took to eat the chocolate cereal reinforcement during the three habituations sessions to the reinforcement.

	Day 1	Day 2	Day 3
Ad Libitum	8185.181 ± 2153.147	318.454 ± 99.294	154.272 ± 23.447
CR	4716.631 ± 1326.374	325.736 ± 79.420	249.210 ± 54.304
Young	3054.812 ± 1274.301	147.125 ± 17.464	96.562 ± 8.366

Figure 34 depicts the global analysis of the latency to find the reinforcement during the three different phases of the ODT. ANOVA demonstrated no statistically significant differences in the acquisition ($F_{(2,43)}$= 0.340, p = 0.714), in the test ($F_{(2,43)}$ = 2.826, p = 0.070), or in the

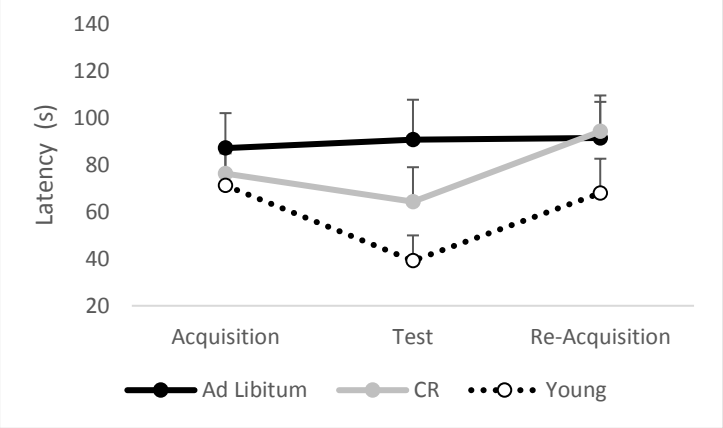


Figure 34: Latency in seconds to find the reinforcement (± SEM) during the four-trial acquisition, the test and the three re-acquisition trials, for each group.

re-acquisition phase ($F_{(2,43)}$ = 1.1013, p = 0.372), indicating that the evolution of the behavior across the three sessions was not different between groups.

The analysis of the number of errors (Figure 35) displayed a similar pattern of results with no statistically significant differences in the acquisition ($F_{(2,43)}$= 1.630, p = 0.208), in the test ($F_{(2,43)}$= 1.907, p = 0.161), or in the re-acquisition ($F_{(2,43)}$= 1.470, p = 0.241), which demonstrates an equivalent performance between groups.

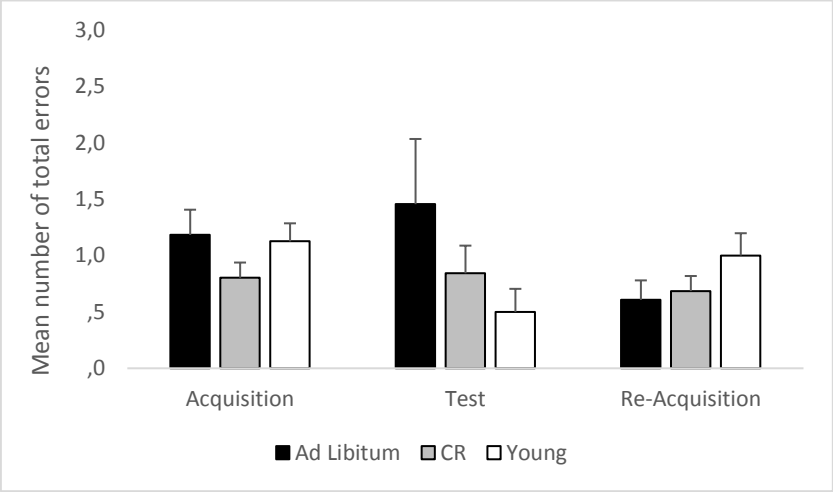


Figure 35: Total mean number of errors (summation of omission and commission error) (± SEM) during the four acquisition trials, the test and the re-acquisition for each group of animals.

Partial discussion for the Olfactory Discrimination Task

The results from the present study demonstrated no differences in the learning and memory of the ODT. However, for the performance of this task, animals were food-deprived for five days, in order to achieve the goal of 80-90% of their initial weight prior to the three sessions of habituation performed to familiarize them with the food reinforcement and the experimental box. All groups became habituated during the three days before the ODT with no significant differences between groups. These results indicated that neither aging nor the food regimen affected the reinforcement intake, as all the animals consumed the cereals during the sessions.

Our data agree with previous experiments (Kraemer & Apfelbach, 2004) in which 28-month-old rats performed as well as young animals in different go/no-go positively reinforced discrimination tasks. The authors concluded that for a variety of odor discrimination duties, the learning ability of aged Wistar *Ad Libitum* rats is normal, indicating that the aptitude to learn an olfactory task does not necessarily decline with age. However, different results have also been found in a flavor memory task that required aged Fischer-344 rats to consume a novel flavor

(Bond et al., 1989). A CR diet did not reduce the age-related deficits regarding the loss of *neophobia* to the new flavored reinforcement when compared the young adult group, which lost their *neophobia* more rapidly. The ODT could be considered to be a strong memory task which involves a survival based behavior for finding food in deprived conditions (Portero-Tresserra., 2013), but, the administration of this task in CR studies could be problematic and controversial. Present results seem to corroborate that CR diet did not change the execution of the animals in the ODT. This may be in part due to the previous fasting procedure to which the animals were subjected in order to reach the percentage of weight necessary to perform positively reinforced tasks. Taking food from an already restricted group of animals seems controversial. In fact, this action can impair the performance of the animals in a task where food is used as a reward (Sohal & Forster, 2014). In humans, caloric deprivation increases responsivity of brain regions implicated in attention and reward to food images, which may contribute to binge eating (Stice et al., 2013). Further factors need to be considered in the interpretation of these results regarding to memory strength or the duration of the retrieval period.

1.5 Olfactory perception test

Figure 36 depicts the latency, in seconds, to find the buried cookie (± SEM) in the cage for each group of animals. The test was carried out 24h after the last trial of the ODT.

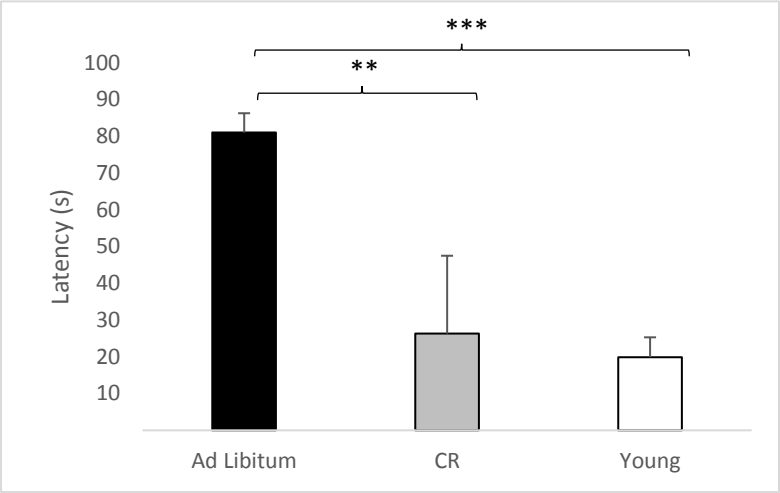


*Figure 36: Latency to find the buried cookie (± SEM) in the olfactory perception test for each group. **p = < 0.01 significant difference between Ad Libitum and CR group. *** p< 0.001 between Ad Libitum and Young group.*

ANOVA indicated statistically significant differences between groups ($F_{(2,43)}$ = 9.214, p < 0.001) and a Post Hoc analysis revealed differences between *Ad Libitum* and Young groups of animals (p = 0.001) and *Ad Libitum* and CR groups (p = 0.002), indicating a worse performance of the aged *Ad Libitum* group.

Partial discussion for the olfactory perception test

Ad Libitum aged animals had a worse execution of the search for the buried cookie. CR seemed to reduce age-decline in this sensorial modality, although no preceding bibliography about CR and olfactory perception was found, which makes it difficult to assess our results. In rats, neuropeptide Y neurons decreases in the anterior olfactory nucleus during aging (Hwang et al., 2001), which could explain the poorer performance of *Ad Libitum* animals. In addition, CR can enhance the neuropeptide Y neurons in the HT (Ferreira-Marques et al., 2016). This neuropeptide is involved in several physiological functions, including increasing food intake, reducing anxiety and stress levels, reducing pain perception and lowering blood pressure. In humans, olfactory function declines with age. There is not only a loss of a sense of smell, but also a loss of the ability to discriminate between smells (Boyce & Shone, 2006). In addition, contradictory results from the ODT and the olfactory perception test lead us to think that given that diverse neuronal systems that participate in the olfactory learning procedure, the aged rat may show deterioration in some learning tasks, but not in others (Kraemer & Apfelbach, 2004). In general, the lack of previous studies in this field keeps us from comparing our results, and further research is needed.

1.6 Object recognition in Y maze

Previous preference test

Before performing the object recognition test, a preference test for one of the arms of the Y maze and the objects was conducted in ten three-month-old male Wistar rats. The arms and the objects were counterbalanced between all the animals. The results shown in Table 13 and 14 are the average time that animals spent in each arm and exploring each object, respectively.

Table 13: Time, spent in each arm was analyzed. The results are expressed as mean in percentage (± SEM).

% time spent in the left arm	% time spent in the right arm
10.427 ± 11.096	14.323 ± 9.763

Table 14: time, spent in the arms where the different objects were (a soda can and a lego construction shape. The results are expressed as mean in percentage (± SEM.)

% time spent in can arm	% time spent in the lego arm
26.064 ± 5.596	26.064 ± 11.243

A T-Test analysis for independent samples was performed and the results indicated no significant preference for any arm ($p = 0.464$) or for any object ($p = 0.703$).

Object recognition in a Y maze

Figure 37 shows the percentage of animals' first choice of arm, (± SEM) (first elected arm once entered the Y maze), during the novel object test. The dotted line represents the chance level. This means that if the elected arm was the one with the novel object, the bars will then be above the line. ANOVA demonstrated no differences between groups ($F_{(2,27)}$= 0.827, p = 0.448). And a T-Test analysis with a fixed value of 50 (represents chance) indicated no differences in the *Ad Libitum* group ($t_{(7)}$= -0.683, p = 0.516), in the CR group ($t_{(11)}$= 1.1793, p = 0.266) or in the Young group ($t_{(9)}$= 0.612, p = 0.555), which implies similar spatial short-term memory between groups in relation to this variable.

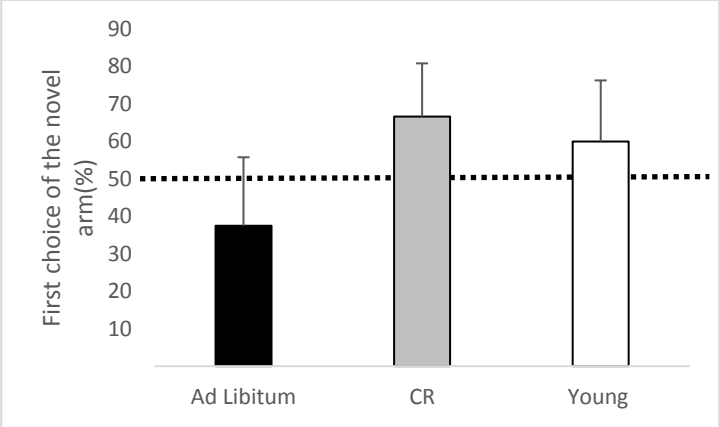


Figure 37: percentage of first choice (± SEM) (first elected arm once entered the Y maze) in the novel arm by the different groups of rats. The black dotted line, at 50%, represents the chance.

Figure 38 depicts the analysis of the short-term memory using the time (in percentage) (± SEM) spent in the novel and the familiar object arms during the test for the three groups of subjects. Dotted line, at 33%, represents the chance of choosing one of three arms of the Y maze. ANOVA demonstrated no significant differences between groups in the novel object arm ($F_{(2,27)}$= 0.963, p = 0.394), or in the familiar object arm ($F_{(2,27)}$= 1.903, p = 0.169). However, a T-Test analysis against a fixed value of 33 (represents the chance level, 33%) demonstrated significant differences in the percentage of time animals would spent in the novel arm compared to the fixed value (33) in the CR group ($t_{(11)}$= 3.277, p = 0.007) and in the Young group ($t_{(9)}$= 2.316, p = 0.046). This indicates that both groups remembered the familiar object and stayed longer in the arm with the new object, showing better memory recall. However, this was not observed for the *Ad Libitum* group ($t_{(7)}$= 1.452, p = 0.190). In relation to the familiar object arm, there were no significant differences from the fixed value in the *Ad Libitum* group ($t_{(7)}$= -0.404, p = 0.698), or in the Young group ($t_{(9)}$= -1.216, p = 0.255), but there were significant differences in the CR group of animals ($t_{(11)}$= -2.433, p = 0.033). The CR group preferred the novel object instead of the old one, demonstrating an effect of diet.

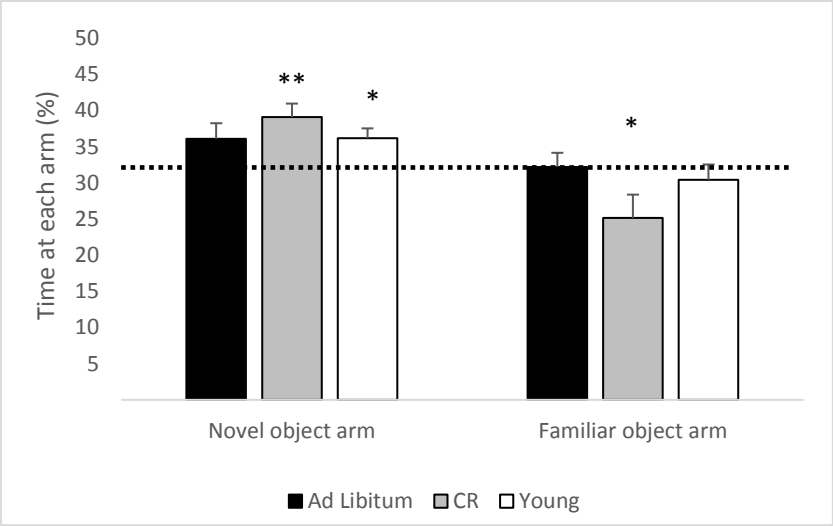


*Figure 38: Time in each arm (± SEM) during the test phase of the object recognition in the Y-maze for each group. Novel object arm **p < 0.01 *p < 0.05. Familiar object arm *p < 0.05. The black dotted line, at 33%, represents chance level.*

In a more exhaustive analysis of the results, the difference in exploration time between novel and familiar objects was evaluated (Figure 39). This was done by extracting the ratio, in percentage of the time the animals spent exploring the novel object in relation to the time they spent exploring the familiar object. The exploration was measured as the amount of time the animal was touching or sniffing the object from at least 2 cm distance. Dotted line represents 0%, which implies an equal time of exploration of both the novel and the familiar object. Bars above the dotted line indicate more exploration of the novel object. Statistical analysis via Welch test showed significant differences between groups ($F_{(2,27)}$= 5.331, p = 0.011), a Post Hoc analysis indicated differences between *Ad Libitum* and Young groups (p = 0.036). A T-Test against a fixed value of 0 (represents chance level) showed no differences in the *Ad Libitum* group ($t_{(7)}$= 1.828, p = 0.110). However, there were significant differences in the CR group ($t_{(11)}$= 2.986, p = 0.012) and in the Young group ($t_{(9)}$= 12.240, p<0.001), proving that both CR and Young groups presented short-term memory recall and preferred the novel object instead of the familiar one.

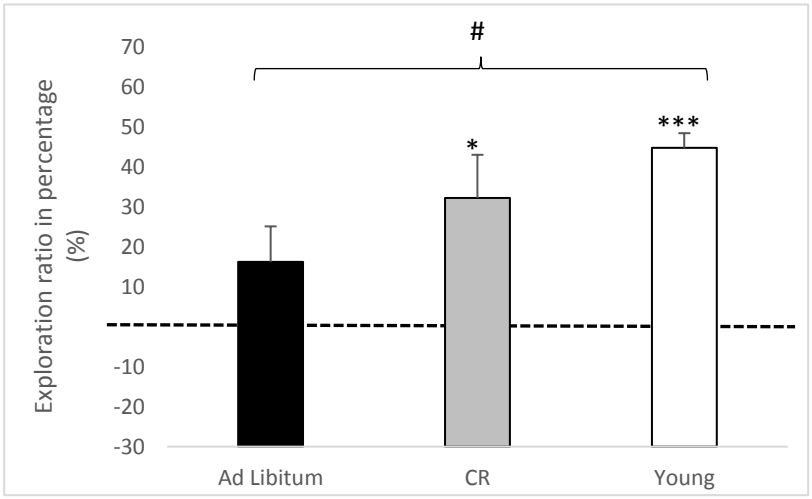


*Figure 39: Object discrimination ratio (time exploring the novel object/time exploring the familiar object) (± SEM) for each group. #p < 0.05 significant difference between Ad Libitum and Young group. *p < 0.05 ***p < 0.001.*

Partial discussion for the object recognition task in Y maze

The protocol carried out during the two-trial recognition task in the Y maze was adapted from the previously used procedure (Dellu et al., 1992) to study memory in aged rats. This task avoids food deprivation that may have non-specific effects and it is based in the innate tendency of rodents to explore the environment. As in the previous study, we also carried out a previous preference test for the arms or the objects and observed no differences between the number of visits to the arms or objects in young control rats, confirming the validity of the task.

The results obtained regarding the first choice of the novel arm did not agree with previous reports (Dellu et al., 1992) of impaired memory retention after 30 min in 18-month-old rats when compared to young rats. However, results regarding the percentage of time at the novel object arm indicated that CR and Young groups explored the novel object for longer, indicating better memory retention. Unfortunately, there are no previous studies that have used both the Y maze and the novel object recognition to evaluate the effect of aging or CR. Nevertheless, there are some studies that assess the effect of CR on a seemingly similar task. For example, a 4-choice multiple maze was used in female Sprague rats in order to analyze the effect of aging on the spatial aspect of the Y maze task. The animals were taught to traverse the maze following a specific order and were then taught to reverse it. The results indicated that aged 23-to-25-month-old animals performance did not differ from the adult 12-to-14 month-old or young 3-month-old groups of animals (Botwinick et al., 1963).

The duration of the visit of the arm is a generally relevant variable for the object exploration (Dellu et al., 1992; Ennaceur & Delacour, 1988). For this reason, we performed a more specific analysis of the time spent exploring the objects (Figure 39). Thus, the results showed a preserved memory in CR and Young animals and an impaired execution in the *Ad Libitum* group. These findings are similar to those obtained in a previous experiment (Shukitt-Hale et al., 2001), which analyzed the effects of novel object recognition memory in the OF in 22-24-month-old F344 rats. The results demonstrated that the ability to build spatial representations of the environment, as well as to detect a change decreased in aged animals, although the object recognition *per se* is not impaired. Further research is needed in order to analyze the CR and aging variables in similar tasks. Nevertheless, by using this task, we demonstrated that CR improves short-term memory (30 min) in aged animals, since the *Ad Libitum* group, but not CR animals experienced age-related difficulties in recognition object memory.

2. Biochemical data

2.1 High Performance Liquid Chromatography

2.1.1 Striatum

Figure 40 indicates the concentration levels of monoamines (NA, DOPAC, DA, HVA, 3MT, 5-HIAA and 5-HT) in the striatum (mean ± SEM; ng/g tissue) for each group of animals. ANOVA showed significant differences in DOPAC ($F_{(2,23)}$= 12.591, p < 0.001). Post Hoc analysis indicated that the differences were between *Ad Libitum* and CR groups (p = 0.001), as well as CR and Young groups (p = 0.001). In DA ($F_{(2\ 20)}$= 65.967, p < 0.001) differences were found between CR and *Ad Libitum* (p= 0.000) and CR and Young groups of animals (p < 0.001). In 3MT ($F_{(2,23)}$= 10.491, p = 0.001) differences were found between CR and *Ad Libitum* (p = 0.025) and CR and Young groups (p = 0.001). CR group presented higher levels compared to both aged *Ad Libitum* and Young animals for all this NTs and metabolites. In contrast, differences in HVA ($F_{(2,23)}$= 3.935, p = 0.034) were only found between CR and Young groups (p = 0.049).

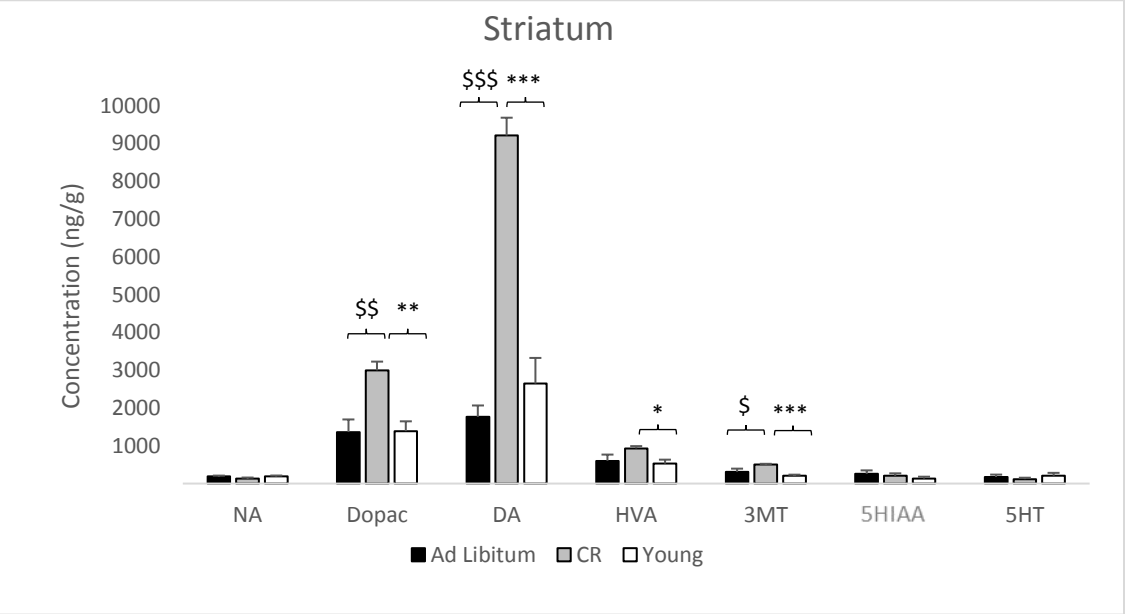


*Figure 40: Concentration levels of monoamines in striatum (mean ± SEM; ng/g tissue) for each group. * < 0.05 **p< 0.01 ***p< 0.001 differences between the CR and Young. $p< 0.05 $$p< 0.01 $$$p <0.001 significant differences between Old CR and Old Ad Libitum groups.*

2.1.2 Hippocampus

Figure 41 depicts the concentration levels of monoamines (NA, DOPAC, DA, HVA, 5-HIAA and 5-HT) in the HPC (mean ± SEM; ng/g tissue) for each group of animals. ANOVA showed differences in NA ($F_{(2,22)}$= 9.303, p = 0.001), of which CR rats expressed higher levels than the Young group (p= 0,001) and *Ad Libitum* animals (p = 0.001). ANOVA also showed differences between groups for 5-HIAA ($F_{(2,22)}$= 4.443, p = 0.024), of which the Young group presented higher levels than *Ad*

Libitum animals (p = 0.040). There were also differences between groups for 5-HT ($F_{(2,22)}$= 3.792, p = 0.038), where a tendency towards significance was observed for the differences between *Ad Libitum* and Young groups (p = 0.057), in which *Ad Libitum* animals tend to present higher levels than the Young group.

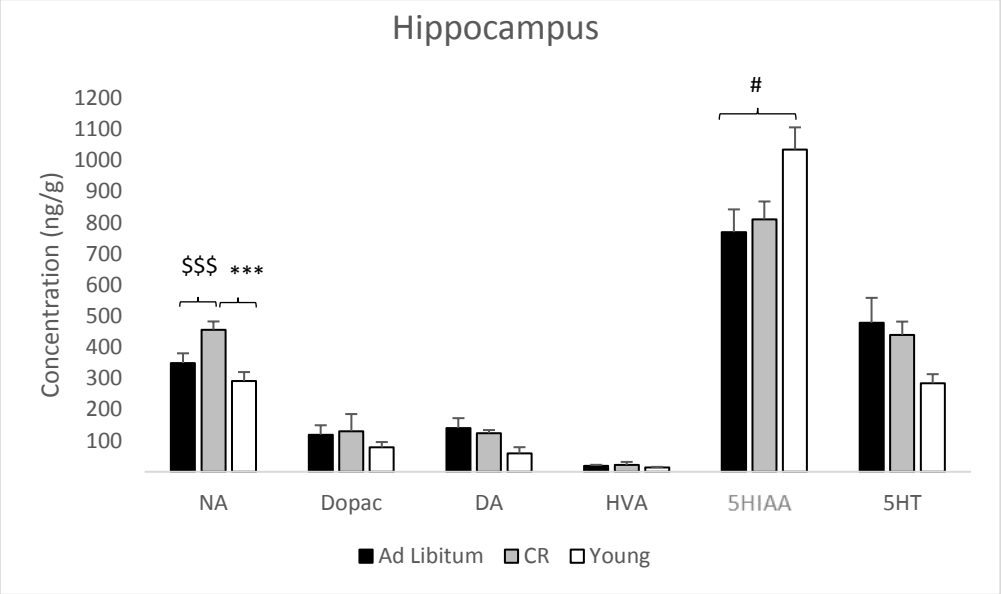


*Figure 41: Concentration levels of monoamines in Hippocampus (mean ± SEM; ng/g tissue) for each group. ***p< 0.001 differences between the CR and Young. $$$p< 0.001 significant differences between Old CR and Old Ad Libitum groups #p< 0.05 differences between Ad Libitum and Young groups.*

2.1.3 Hypothalamus

Figure 42 depicts the concentration levels of monoamines (NA, DOPAC, DA, HVA, 5-HIAA and 5-HT) in the HT (mean ± SEM; ng/g tissue) for each group of animals. ANOVA indicated differences in DA ($F_{(2,21)}$= 3.859, p = 0.037), of which Young animals presented more elevated levels compared to the *Ad Libitum* group (p = 0.037). Differences were also found in 5-HT ($F_{(2,21)}$ = 3.868, p = 0.037) and Post Hoc analysis confirmed that the Young group levels were higher than that of *Ad Libitum* animals (p = 0.040).

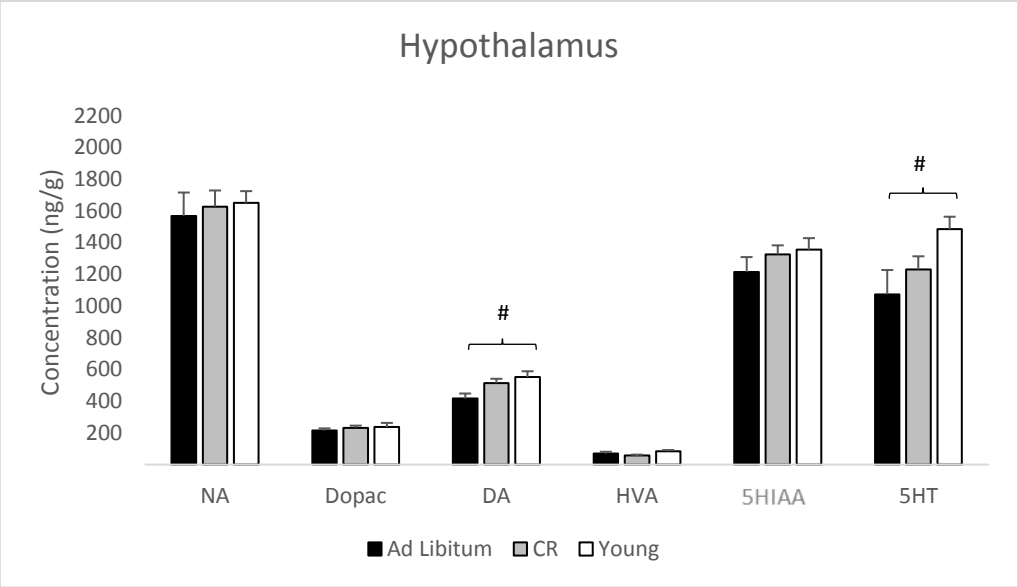


Figure 42: Concentration levels of monoamines in Hypothalamus (mean ± SEM; ng/g tissue) for each group. #p< 0.05 significant differences between the Ad Libitum and Young groups.

2.1.4 Occipital cortex

Figure 43 depicts the concentration levels of monoamines (NA, DOPAC, DA, HVA, 5-HIAA and 5-HT) in the Occipital cortex (mean ± SEM; ng/g tissue) for each group of animals. ANOVA indicated differences in DA ($F_{(2,22)}$= 4.339, p = 0.026) and in 5-HT ($F_{(2,21)}$= 3.897, p = 0.036). Posterior Post Hoc analysis revealed that in DA, the Young group had significant higher levels than the *Ad Libitum* animals (p = 0.026), as well as in 5-HT levels, which were higher in Young animals compared to *Ad Libitum group* (p = 0.022).

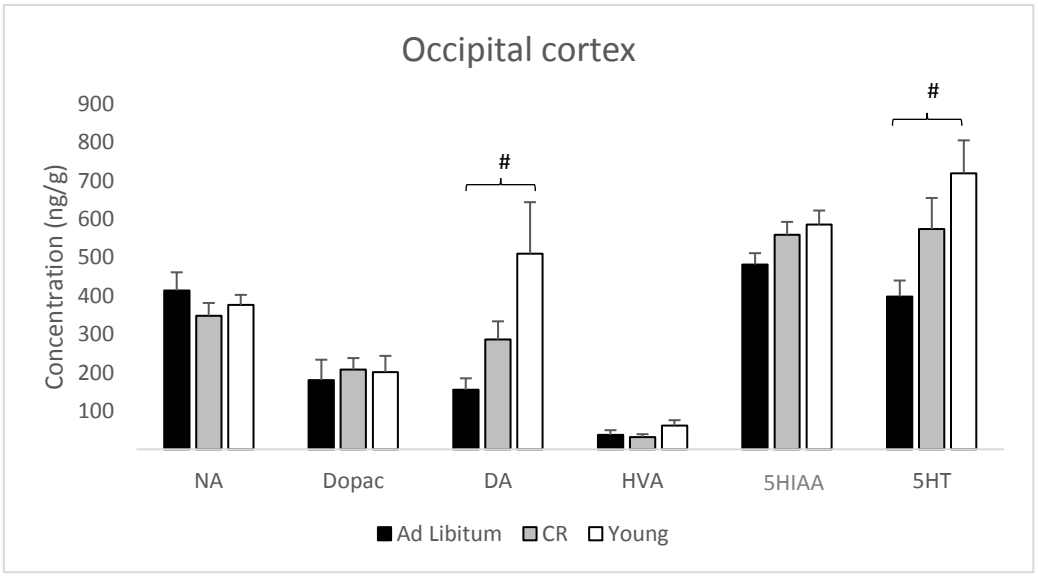


Figure 43: Concentration levels of monoamines in Occipital cortex (mean ± SEM; ng/g tissue) for each group. #p< 0.05 significant differences between Ad Libitum and Young groups.

2.1.5 Frontal cortex

Figure 44 shows the concentration levels of monoamines (NA, DOPAC, DA, 5-HIAA and 5-HT) in the FC (mean ± SEM; ng/g tissue) for each group of animals. ANOVA demonstrated differences in DOPAC ($F_{(2,22)}$= 10.801, p = 0.001) and DA ($F_{(2,23)}$= 24.147, p < 0.001). Post Hoc analysis indicated that in DOPAC Young animals showed higher levels than *Ad Libitum* (p = 0.011) and CR animals (p = 0.001), similar to DA levels, in which Young animal levels were higher, compared to both *Ad Libitum* and CR groups (p < 0.01). Finally, ANOVA also showed significant group differences in 5-HT ($F_{(2,21)}$= 5.056, p = 0.016), which was increased in CR rats compared to *Ad Libitum* groups (p = 0.013).

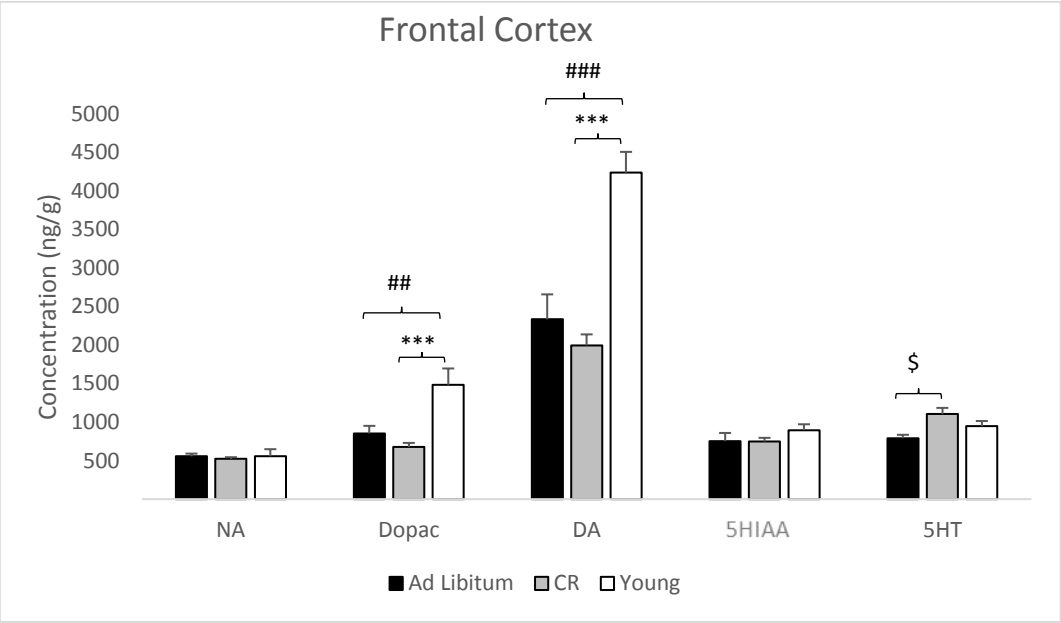


*Figure 44: Concentration levels of monoamines in Frontal cortex (mean ± SEM; ng/g tissue) for each group. ***p< 0.001 difference between CR and Young group of animals. ##p< 0.01 ###p< 0.001 differences between Ad Libitum and the Young. $p< 0.05 differences between Ad Libitum and CR.*

Table 15 indicates the concentration of neurotransmitters and their metabolites (mean ± SEM; ng/g tissue) for each brain region explained in the previous figures:

Table 15: Concentration of neurotransmitters and metabolites (mean ± SEM; ng/g tissue) for each brain region in Young rats, Ad Libitum-*fed old rats (Ad Libitum) and old rats fed with a restrictive diet (CR). *p< 0.05, **p< 0.01, ***p< 0.001 indicates significant differences between the CR and Young groups. #p< 0.05, ##p< 0.01, ###p< 0.001 indicates significant differences between the Old AL and the Young groups. $p< 0.05, $$p< 0.01, $$$p< 0.001 indicates significant differences between Old CR and Old Ad Libitum groups.*

	Ad Libitum	**CR**	**Young**
Noradrenaline			
Striatum	192.135 ± 18.672	135.446 ± 23.712	192.761 ± 19.605
Hippocampus	349.478 ± 31.114 $$$	456.581 ± 26.419 ***$$$	291.618 ± 28.767 ***
Hypothalamus	1568.895 ± 147.151	1627.547 ± 101.497	1650.924 ± 73.879
Frontal cortex	555.805 ± 35.505	525.065 ± 19.459	556.010 ± 92.327
Occipital lobe	413.867 ± 47.395	348.207 ± 33.189	376.136 ± 26.311
DOPAC			
Striatum	1363.466 ± 333.459 $$	2997.00 ± 230.797 **$$	1388.565 ± 258.772**
Hippocampus	119.192 ± 30.258	130.411 ± 55.084	78.333 ± 17.223
Hypothalamus	214.200 ± 14.142	232.111 ± 13.746	237.591 ± 25.396
Frontal cortex	852.798 ± 98.546 ##	677.124 ± 52.606 ***	1485.707 ± 210.258 ***##
Occipital lobe	180.269 ± 53.294	207.679 ± 30.126	201.522 ± 42.029
Dopamine			
Striatum	1764.294 ± 302.623 $$$	9212.092 ± 471.228 ***$$$	2647.347 ± 677.917 ***
Hippocampus	140.892 ± 31.394	124.135 ± 9.876	58.917 ± 20.095
Hypothalamus	416.541 ± 31.795 #	513.163 ± 28.172	552.117 ± 36.727 #
Frontal cortex	2333.709 ± 324.115 ###	1996.052 ± 142.532 ***	4237.610 ± 269.524 ***###
Occipital lobe	154.935 ± 30.086 #	286.145 ± 47.307	509.976 ± 133.939 #
HVA			
Striatum	600.278 ± 166.878	931.653 ± 59.198 *	527.636 ± 105.070 *
Hippocampus	18.999 ± 3.370	21.865 ± 9.856	13.892 ± 1.908
Hypothalamus	68.847 ± 12.163	57.071 ± 5.842	84.108 ± 7.801
5-HIAA			
Striatum	257.608 ± 91.078	205.573 ± 65.008	134.076 ± 47.403
Hippocampus	769.746 ± 73.130 #	811.203 ± 57.102	1035.308 ± 71.069 #
Hypothalamus	1215.522 ± 93.372	1326.409 ± 56.529	1355.439 ± 72.886
Frontal cortex	753.472 ± 105.891	748.679 ± 47.238	895.538 ± 76.400
Occipital lobe	481.695 ± 29.803	559.256 ± 33.353	585.594 ± 36.642
Serotonin			
Striatum	175.131 ± 61.918	117.517 ± 37.162	209.444 ± 74.050
Hippocampus	478.494 ± 80.229	439.854 ± 42.657	284.235 ± 29.305
Hypothalamus	1074.273 ± 152.989 #	1231.535 ± 82.288	1484.542 ± 78.960 #
Frontal cortex	789.803 ± 45.388 $	1103.803 ± 80.350 $	946.002 ± 68.102
Occipital lobe	398.101 ± 41.732 #	573.772 ± 81.164	719.158 ± 85.861 #

Partial discussion for the HPLC analysis

In our experiment, the results from the dopaminergic system demonstrated that a reduction of 30% in the caloric intake significantly enhanced levels of precursor DOPAC, DA, as well as metabolites HVA and 3MT in the striatum. In general, our results confirmed that levels of DA and its metabolites tend to decline with age (Koprowska et al., 2004; Luine et al., 1990), nevertheless the data also showed that CR might be able to reverted it. The same results have been described in studies using a 30% CR diet for 6 month in rhesus monkeys (Maswood et al., 2004). However, a different study (Del Arco et al., 2011) did not agree with our results, since no differences were found in the levels of DA in the PFC in 24-month-old CR aged rats compared to control 6-month-old rats. Moreover, an age-related negative progression of DA levels were detected in the FC, which is consistent with what has been previously described (Eppinger et al., 2011; Koprowska et al., 2004; Lee et al., 1994; Míguez et al., 1999; Ponzio et al., 1982). Furthermore, levels of DA and the dopaminergic metabolite DOPAC were stable in the HPC, in disagreement with previous reports (Del Arco et al., 2011). Nevertheless, no general conclusion can be drawn about DA and metabolites level, because different and contradictory results have been found. In addition, scarce information about the DA levels in the striatum was found, which was the only area in which CR seem to have effect in the DA transmission in the present study.

Regarding the levels of serotoninergic NT and metabolites, no differences were found in the striatum. However, the present experiment demonstrated that aged animals presented a general reduction of the expression of 5-HT in the HT and Occipital cortex, which was reverted by the application of a CR diet, as previous researches have demonstrated (Sirviö, et al., 1994; Stemmelin et al., 2000). In addition, similar findings regarding levels of 5-HT in FC have been previously described after prolonged 7 to 21 days fasted diet in humans (Michalsen, 2010). Regarding the 5-HIAA metabolite, the overall literature is controversial. As far as we know, only one author (Esteban et al., 2010) has found a decrease of hippocampal 5-HIAA in aged animals, which is similar to our findings. Other studies have indicated equivalent levels of 5-HIAA (Godefroy et al., 1987; Koprowska et al., 2004; Luine et al., 1990; Ponzio et al., 1982; Stemmelin et al., 2000) or even higher levels (Lee et al., 1994; Sirviö et al., 1994).

Furthermore, CR enhanced the noradrenergic neurotransmission. Specifically, levels of NA in the HPC compared to aged and Young animals. In contrast with our results, previous studies on aging demonstrated similar levels of NA in HPC (Lee et al., 1994; Nakamura & Ohno, 1995; Ponzio et al., 1982). No other differences were found in levels of NA NT regarding the rest of brain areas. Studies have demonstrated similar levels of this neurotransmitter in aged animals in the HT

(Carfagna et al., 1985; Tanila et al., 1994) and PFC (Lee et al., 1994) as our results indicated. However, it is difficult to compare our results with other studies, since no data regarding NA transmission and CR animals was found.

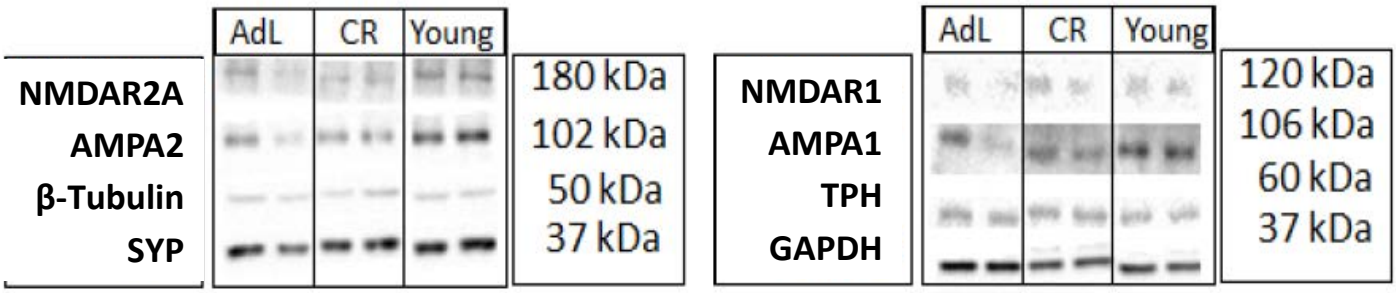
In general, the HPLC findings support the hypothesis that significant decrease in brain monoamines occur during the aging process, which is in agreement with previous studies (Koprowska et al., 2004; Luine et al., 1990). Moreover, increased DA, DOPAC and HVA in animals with a CR diet has also been previously described (Maswood et al., 2004). In addition, enhanced levels of 5-HT in PFC have as well been reported (Michalsen, 2010). However, previous studies have not focused on NA and CR. Further research must be carried out in order to clarify controversial or inconclusive data.

2.2 Western Blotting

2.2.1 Hippocampus

Figure 45a depicts the band intensity for glutamatergic receptors NMDA and AMPA subunits, synaptic protein SYP and catecholamine precursor TPH in the HPC. β-Tubulin and GAPDH were used as loading control. Figure 45b shows histograms and the change (in percentage) in the intensity of the protein bands taking the Young control group as 100%. Values represent mean (± SEM). ANOVA indicated significant differences in AMPA1 ($F_{(2,11)}$= 5.387, p = 0.023) and AMPA 2 ($F_{(2,13)}$ = 4.345, p = 0.036) subunits. Post Hoc analysis detected differences between the Young group than *Ad Libitum* in AMPA1 (p = 0.028) and in AMPA2 (p = 0.05). In both cases *Ad Libitum* old rats expressed less AMPA subunits than the Young group. No differences between groups in NMDAr subunits (NMDAR2A ($F_{(2,15)}$ = 0.428, p= 0.660); NMDAR1 ($F_{(2,15)}$ = 0.496, p= 0.618), TPH($F_{(2,15)}$ = 0.291, p= 0.751) and SYP ($F_{(2,15)}$ = 0.091, p= 0.913) were found.

a)



b)

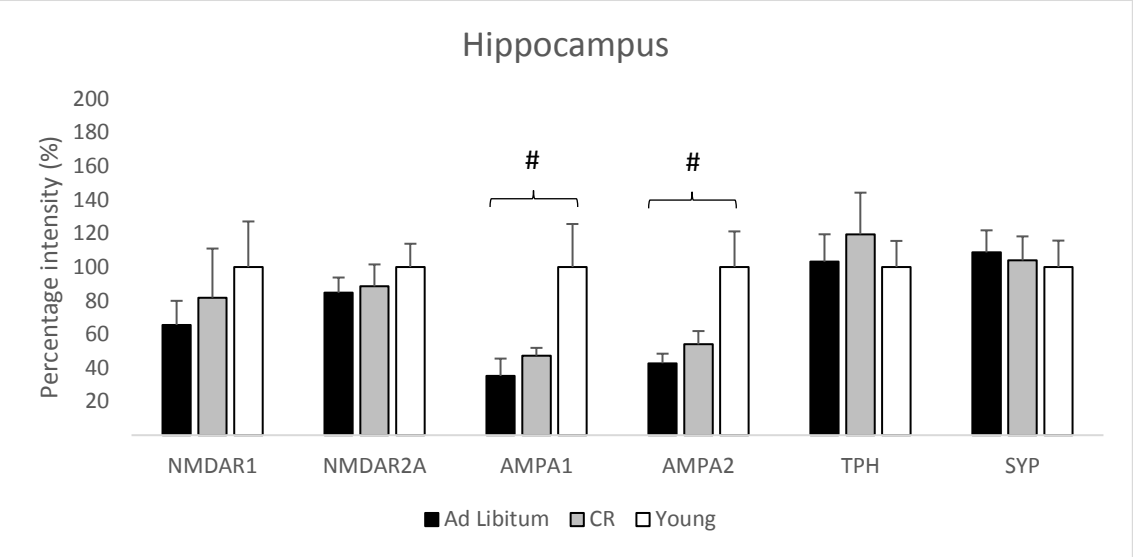


Figure 45: Representative Western Blot analysis for glutamatergic receptors NMDAR2, NMDAR1, AMPA2, AMPA1 subunits, synaptic protein SYP and catecholamine precursors TPH in HPC region of aged Ad Libitum (AdL), old calorie restricted (CR) and young rats. A) Histograms of the integrated density bands of proteins represented by the percentage of change taking intensity in young rats as 100%. B) Values are mean ± SEM of the 3 groups. #p < 0.05 in AMPA1 and #p < 0.05 in AMPA2 Ad Libitum compared to Young group of animals.

Partial discussion for Western Blot analysis in the Hippocampus

On the one hand, the general decreased levels of AMPA1 in the HPC observed in the *Ad Libitum* group are in line with results reported in previous studies (Adams et al., 2008; Newton et al., 2008; Shi et al., 2007). However, there is one study (Eckles-Smith et al., 2000) that did not show differences in the AMPA1 levels between CR, *Ad Libitum* and young animals. Moreover, different and controversial results have been found in the case of AMPA2. In general, CR is able to attenuate the loss of AMPA2 in old rats observed in *Ad Libitum* animals (Adams et al., 2008; Shi et al., 2007), but some studies (Newton et al., 2008; Shi et al., 2007) have demonstrated that both aged CR and *Ad Libitum* animals presented decreased levels compared to control Young animals. All these results have led to controversial and inconclusive suppositions. Despite all this, previous findings are in the same line as our results and this may indicate that CR could help prevent the age-decline in both AMPA receptors subunits (Adams et al., 2008).

On the other hand, the lack of differences between groups in the levels of NMDAr subunits in the HPC is not supported by previous studies (Eckles-Smith et al., 2000), which have indicated that this receptor and its subunits tend to decrease during aging and that CR diet could prevent the drop in the NMDAR1 subunit (Adams et al., 2008; Monti et al., 2004; Newton et al., 2008; Shi et al., 2007). However, other studies (Monti et al., 2004) failed to demonstrate any beneficial effects of CR on NMDAr subunits. Furthermore, some authors (Adams et al., 2008; Newton et al., 2008; Shi et al., 2007) have reported a reduction in the levels of NMDAr subunit in the *Ad*

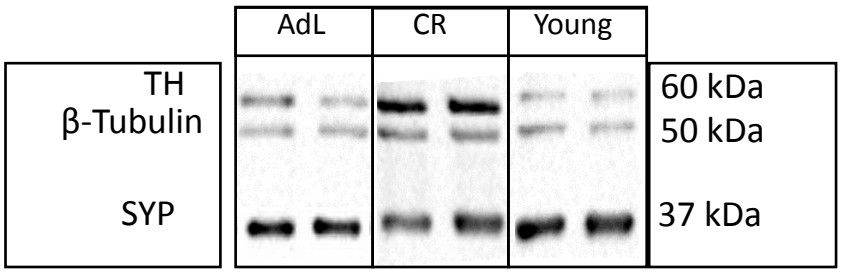
Libitum group, while the CR group maintained stable levels similar to those of the control young group. Overall, as indicated above, different findings of the NMDAr have been obtained and our results did not confirm an effect of neither age nor dietary intervention on these receptors.

Finally, previous experiments (Adams et al., 2008; Singh et al., 2015) have indicated an increased expression of TPH and SYP proteins in the aged CR group in the HPC compared to *Ad Libitum* animals and also a decrease in the hippocampal SYP of aged rats *Ad Libitum* (Portero-Tresserra et al., 2018). However, we were unable to corroborate previous findings based on the results obtained in our experiment. Therefore, our results suggested that neither age nor the dietary intervention modified protein levels of in the HPC, striatum, or FC. This will be more thoroughly discussed in the following sections (Figures 46b and 47b)

2.2.2 Striatum

Figure 46a shows the band intensity of synaptic proteins SYP and TH in the striatum. β-Tubulin and GAPDH were used as loading controls. The Figure 46b depicts the histograms of the change in the intensity (in percentage) of the protein band in relation to the young control group, which was assigned the value as the 100%. Values represents mean ± SEM. ANOVA indicated significant differences in TH ($F_{(2,17)}$= 16.818, $p < 0.001$) between groups. Post Hoc analysis revealed that higher levels found for the CR group were significantly different from those of the Young ($p = 0.003$) and *Ad Libitum* ($p < 0.001$) groups. No differences were found between subjects in the SYP levels ($F_{(2,17)}$= 0.007, p= 0.751).

a)



b)

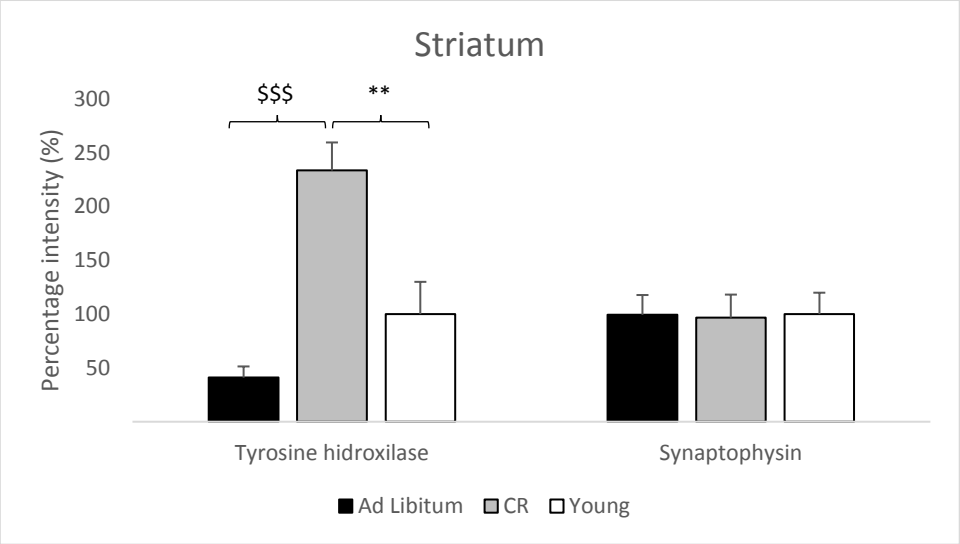


*Figure 46: Representative Western Blot analysis for catecholamine precursors TH and synaptic protein SYP in the striatum region of aged Ad Libitum (AdL), old calorie restricted (CR) and young rats. A) Histograms of the integrated density bands of proteins represented by the percentage of change taking intensity in young rats as 100%. B) Values are mean ± SEM of the 3 groups. $$$p < 0.001 between Ad Libitum and CR groups and **p < 0.01 between Young and CR groups.*

Partial discussion for Western Blot analysis in the striatum

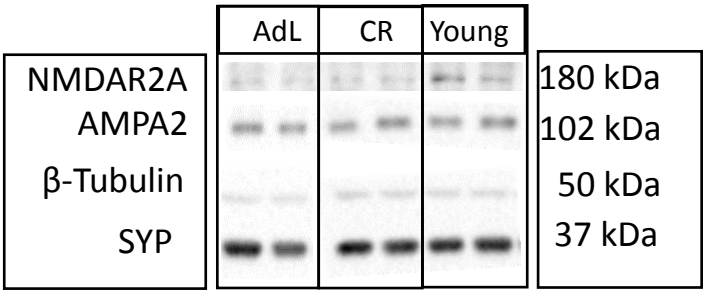
The higher expression of TH in the CR group fully correlates with the upper levels of DA and metabolites obtained via HPLC found in the striatum area (Figure 40). Our results are also consistent with those obtained by previous studies (Holmer et al., 2005; Kastman et al., 2012).The maintenance of DA neurons can counteract the development of neurodegenerative illness such Parkinson and Huntington, in which dopaminergic neurons in the substantia nigra and in the striatum succumb (Mattson & Magnus, 2006). Other authors have found similar results, for example, IF (feeding every other day) applied to 24-month-old CR animals delayed the normal age-associated loss of striatum DA2 receptors (Roth et al., 1984), although this decrease seemed to disappear when analyzed in 30-month-old CR animals. Furthermore, 26-to-28-month-old F344 female rats showed, via in vivo electrochemistry, a greater evoked striatal DA overflow in amplitude and duration when compared to the *Ad Libitum* aged animals (Diao et al., 1997). Taken together, these results seem to suggest that CR diet improves the age-related decline of DA neuronal function and protect against neurodegenerative diseases through the upregulation of its activity in the TH.

Finally, no previous studies on the expression of SYP protein in this brain area with a CR diet were found. Therefore, no further comparisons were established between our results and previous literature.

2.2.3 Frontal cortex

Figure 47a shows the band intensity of glutamatergic receptors NMDAR2A, NMDAR1, AMPA1 and AMPA2 subunits, synaptic protein SYP and catecholamine precursors TH and TPH in the FC. β-Tubulin and GAPDH were used as loading control. Figure 47b depicts the histograms of the change in the intensity (in percentage) of the protein band in relation to the young control group, which was assigned the value as the 100%. Values are represented as mean ± SEM. ANOVA indicated significant differences in TH ($F_{(2,18)}$= 4.426, p = 0.027). Posterior analysis revealed differences between Young and CR (p = 0.027) groups, indicating higher expression in Young animals. No statistical significant differences between groups were found in NMDAr (NMDAR2A ($F_{(2,14)}$= 0.029, p= 0.972) NMDAR1 ($F_{(2,17)}$= 1.071, p= 0.365) or AMPAr (AMPA1 ($F_{(2,16)}$= 0.633, p= 0.544) AMPA2 ($F_{(2,14)}$= 1.370, p= 0.286), TPH ($F_{(2,14)}$= 0.703, p= 0.512) and SYP ($F_{(2,18)}$= 1.071, p= 0.365).

a)



b)

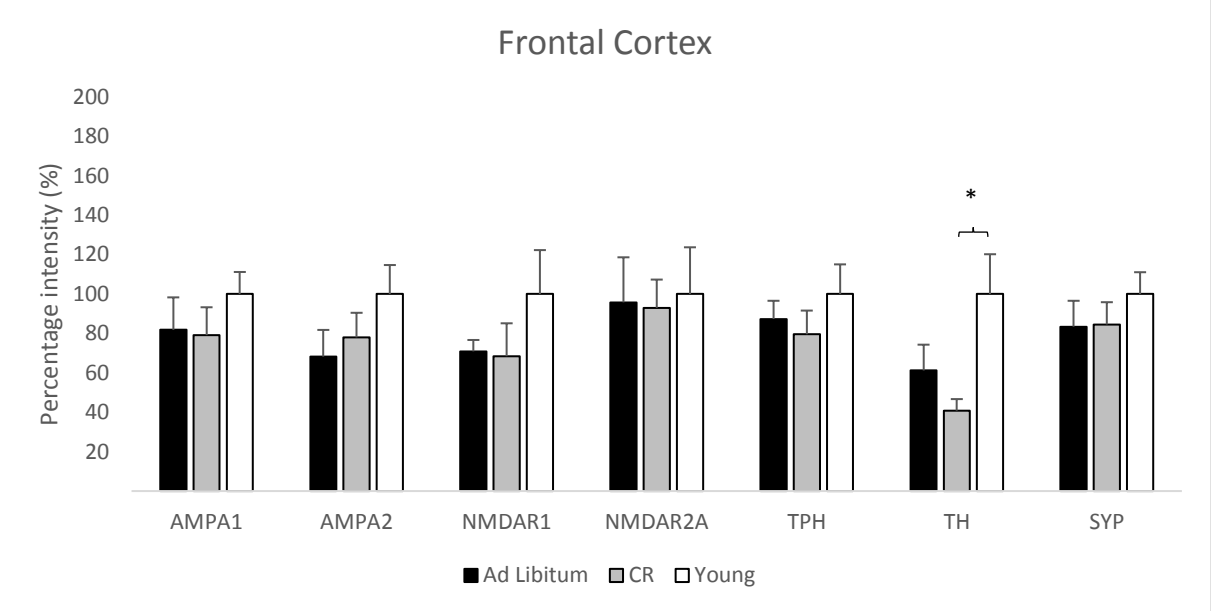


*Figure 47: Representative Western Blot analysis for glutamatergic receptors NMDAR2A, NMDAR1, AMPA2, AMPA1 subunits, synaptic protein SYP and catecholamine precursors TPH and TH in the FC region of aged Ad Libitum (AdL), old calorie restricted (CR) and young rats. A) Histograms of the integrated density bands of proteins represented by the percentage of change taking intensity in young rats as 100%. B) Values are mean ± SEM of the 3 groups. *p < 0.05 between CR and Young groups of animals.*

Partial discussion for Western Blot analysis in the frontal cortex

Our results did not show an effect of age or CR on NMDAr and AMPAr subunits in the FC, which did not support previous reports of a decreased expression of NMDAr in the FC (Castorina et al., 1994). However, to our knowledge, no studies have directly assessed the trafficking of AMPARs in animal models of normal aging and CR in the FC in rats.

The enhanced levels of the precursor of DA, TH, which were observed in the young group of animals in the FC, are partially aligned with the higher levels of DA observed in the same group of animals quantified by HPLC (Figure 44). This demonstrates that the Young group presented an elevated pool of dopaminergic NTs. In agreement with our findings, previous studies (Chisholm et al., 2013) also indicated lowered levels of TH in the HPC of aged 21-month-old male F344 rats. The authors suggested that this effect might be due to the general decrease of testosterone during aging in males, because the reduction of TH was not significant in female aged rats. This hypothesis leads to think of a protective effect of ovarian hormones during aging on dopaminergic function.

2.3 Blood plasma analysis

2.3.1 Health related blood plasma analysis

All the variables related to health and welfare of animals are depicted in Table 16. ANOVA did not detect significant differences between groups in the levels of Cholesterol ($F_{(2,22)}$= 3.179, p = 0.061) and Triglycerides ($F_{(2,22)}$= 1.811, p = 0.187). Moreover, regarding LDL ($F_{(2,22)}$= 17.174, p < 0.001), total protein ($F_{(2,22)}$= 13.325, p < 0.001), calcium ($F_{(2,22)}$ = 42.801, p < 0.001) and albumin ($F_{(2,22)}$ = 21.876, p < 0.001) demonstrated that young animals presented lower levels compared to CR (LDL p = 0.001, total protein p < 0.001, calcium p = 0.017 and albumin p < 0.001) and *Ad Libitum* groups (LDL p < 0.001, total protein p = 0.029, calcium p < 0.001 and albumin p = 0.018). Furthermore, ANOVA showed differences in HDL levels ($F_{(2,22)}$= 14.376, p < 0.001), in which CR presented significantly diminished levels compared to Young (p < 0.001) and *Ad Libitum* groups (p = 0.001). In addition, levels of ALP ($F_{(2,22)}$= 3.609, p = 0.044), were increased in CR animals compared to *Ad Libitum* group (p = 0.05).

*Table 16: Concentration of plasma analysis (mean ± SEM; each concentration level is expressed on the first row of the table) for each group of animals (Young rats, Ad Libitum-fed old rats and old rats fed with a restrictive diet, CR). *p< 0.05, **p< 0.01, ***p< 0.001 indicates significant differences between the CR and Young groups. #p< 0.05, ##p< 0.01, ###p< 0.001 indicates significant differences between the Old Ad Libitum and the Young groups. $p< 0.05, $$p< 0.01, $$$p< 0.001 indicates significant differences between Old CR and Old Ad Libitum groups.*

	Ad Libitum	**CR**	**Young**
Cholesterol (mg/dL)	103.985 ± 6.563	79.83 ± 9.114	78.462 ± 5.066
Triglycerides (mg/dL)	103.085 ± 7.860	120.31 ± 20.456	81 ± 5.118
LDL (mmol/L)	0.815 ± 0.028 *###*	0.712 ± 0.057 *****	0.425 ± 0.035 *###* *****
HDL (mmol/L)	1.197 ± 0.127 *$$$*	0.732 ± 0.045 ***** *$$$*	1.22 ± 0.056 *****
Total Protein (g/dL)	5.978 ± 0.178 *#*	6.452 ± 0.184 *****	5.268 ± 0.115 *#* *****
Calcium (mg/dL)	9.485 ± 0.208 *###*	10.13 ± 0.081 *	8.262 ± 0.168 *###* *
Albumin (g/dL)	2.675 ± 0.147 *#*	3.178 ± 0.114 *****	2.168 ± 0.059 *#* *****
ALP (UI/L)	62.125 ± 12.214 *$*	114.85 ± 17.047 *$*	80.836 ± 9.432

Partial discussion for the health-related blood plasma data

The results presented in the Table 16 indicated that in general, cholesterol and triglycerides levels were similar between groups. Unfortunately, we cannot fully compare our results with previous ones, as there is little information on these variables in rodent studies. For this reason, experiments on humans and monkeys will be discussed. Preceding studies (Cefalu et al., 2004) in monkeys support our results regarding triglycerides levels. Interestingly, our experiment showed that, although the difference between total cholesterol levels were not statistically

significant, the CR group levels were closer to that of the Young group, in agreement with previous data (Masoro, 2007). This demonstrated that CR might revert the enhanced levels of triglycerides in the aged group. Regarding LDL and HDL levels, different studies have found divergent results. For example, monkeys fed with low fat diets presented higher HDL levels (Roth & Polotsky, 2012). Previous findings (Fontana et al., 2004) in human research demonstrated that a prolonged (8 to 16 years) CR diet decreased LDL levels and increased HDL cholesterol. In addition, results from the Biospherian study, which demonstrated decreased levels of HDL in the CR group (Walford et al., 1992), were more similar to our outcome. However, the differences between studies can potentially be explained by differences in the dietary composition (Speakman & Mitchell, 2011) and in the percentage and time of the CR intervention.

The total protein in serum, which is composed of albumin (35 to 50% of total protein), globulin and other proteins, collectively serves to various functions, including maintaining colloid osmotic pressure, as well as acting as enzymes, antibodies and hormones (Zaias et al., 2009). This protein has a special role in general binding of water, cations, fatty acids and hormones. In addition, its main function is to regulate the oncotic pressure of blood, which is necessary for the well-balanced level of liquids in the peripheral system. Previous studies (Torbert, 1935) demonstrated that fasting could modify the levels of total protein, which would help explain the higher levels found in the CR group. This group endured a feeding schedule in which food was presented one time per day, presenting a “fasting-like” behavior the rest of the time.

Moreover, calcium levels were increased in the CR group compared to Young and *Ad Libitum* animals. In addition, CR group presented the highest levels of corticosterone (Figure 48). Previous studies (McBroom & Weiss, 1973) in F344 rats demonstrated a direct relation between brain calcium and age. Moreover, a link between elevated glucocorticoids and increased extracellular calcium levels, which may affect the activation of NMDA receptors by glutamate, has also been described (Armanini et al, 1990). It seems that brain levels of glucocorticoids, such as corticosterone, inhibits glutamate intake by glial cells, causing neuron death by a persistent influx of calcium (Armanini et al., 1990). These results can be explained via the upregulation of calcium due to aging, as well as due to the elevated levels of glucocorticoids in blood.

Furthermore, ALP indicated no abnormal levels compared to basal information from Charles River rats provider. This enzyme seems to play a main role in bacterial protection and mediation of inflammation, which implies that animals’ health is generally good in terms of the liver, gall bladder and bones. In particular, low levels of this enzyme are related to malnutrition, anemia and hypothyroidism (Lum., 1995; Sharma et al., 2014). These findings demonstrated that the CR

rats were in good health and with no malnutrition due to the diet regimen, similar to the other groups of animals, although CR group presented higher level than *Ad Libitum* animals, but both groups demonstrated normal levels.

2.3.2 Glucose

Figure 48 describes levels of plasma glucose (mg/dL) in the three experimental groups. ANOVA demonstrated differences between groups ($F_{(2,22)}$= 1.733, p < 0.001). Post Hoc analysis showed differences between the Young group compared to CR (p < 0.001) and *Ad Libitum* groups (p = 0.002). In both cases, the Young group showed lower glucose levels than the aged animals.

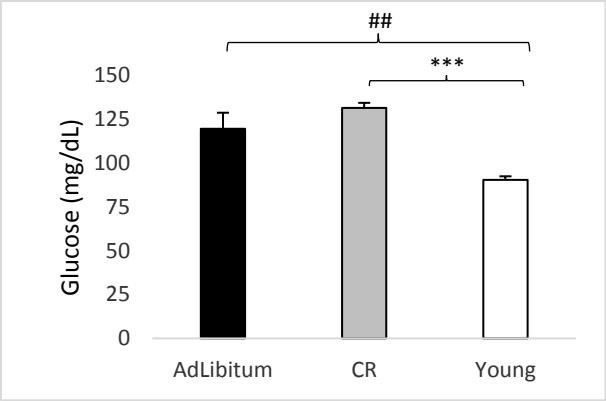


*Figure 48: Plasma levels of Glucose (mg/dL) in Ad Libitum, CR and Young groups of animals. Values are mean ± SEM). ## p < 0.01 between Ad Libitum and Young groups and ***p < 0.001 between CR and young animals.*

Partial discussion for blood glucose levels

The effect age seems to have on the blood glucose concentration was not reverted by CR. Aged animal presented higher levels of blood glucose, as previously demonstrated (Sonntag et al., 2005). However, controversial findings regarding glucose levels have been reported. Studies on primates (Mattison et al., 2017) indicated that CR and *Ad Libitum* up to 23-year-old animals presented an age-related increased glucose level. In contrast, other study on rhesus monkeys (Kemnitz, 2011) with long term (10 years) CR diets indicated that, while some animals maintained fasting plasma glucose levels in the normal range, others presented hyperinsulinemia and moderately elevated glucose levels, and there were even some that became diabetic. Similarly, experiments (Wan et al., 2010) in young 3-month-old rats with IF revealed that those animals presented significantly lower levels of plasma glucose compared to *Ad Libitum* animals. In general, there is not a consensus regarding glucose and CR, but it is supposed that the CR diet induces a decline in insulin, glucose and reproductive hormones during aging (Barzilai & Bartke, 2009), which does not fully support our results. Nevertheless, we need to consider the possibility that it is necessary to take multiple samples of insulin and glucose during the experimentation in order to eliminate further variables such as fasting period, anxiety or circadian rhythm, which may have affected the results.

2.3.3 Leptin and insulin

Figure 49 shows Leptin levels (pg/mL) in blood plasma in the three groups of animals. ANOVA demonstrated differences between groups ($F_{(2,22)}$= 26.281, p < 0.001). Post Hoc analysis showed differences between the *Ad Libitum* and the Young group (p < 0.001) and CR group (p < 0.001). Levels of leptin were higher in *Ad Libitum* animals compared to Young and CR groups.

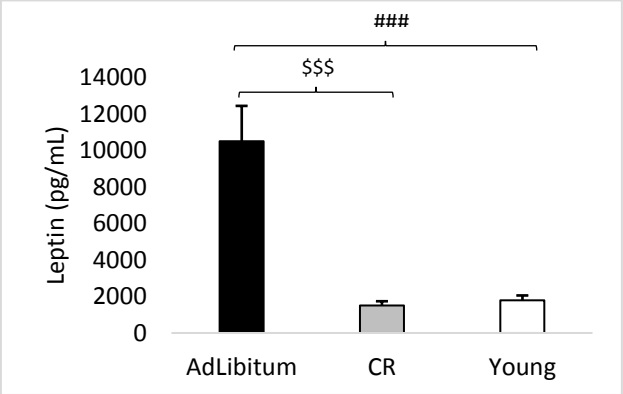


Figure 49: Plasma levels of Leptin in pg/mL in Ad Libitum, CR and Young groups of animals. Values are mean ± SEM). ### <= 0.001 between Ad Libitum and Young group and $$$p < 0.001 between Ad Libitum and Young group of animals.

Figure 50 indicates the levels of plasma insulin (µg/mL)**.** ANOVA showed no differences between groups ($F_{(2,22)}$= 0.139, p = 0.718), this variable was not modified by age or CR diet.

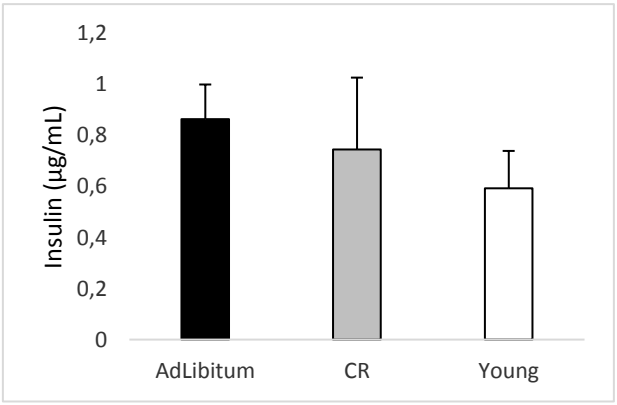


Figure 50: Plasma levels of Insulin (µg/mL) in Ad Libitum, CR and Young groups of animals. Values are mean ± SEM.

Partial discussion for leptin and insulin levels

Leptin and insulin levels are suitable predictors of global adiposity status (Weigle et al., 1997). In general, leptin levels increase with age and CR is able to attenuate this effect. Our results agreed with those obtained by previous studies (Amitani et al., 2017). In addition, other studies have also confirmed that both short-term (Imbeault et al., 2004) and long-term (Wisse et al., 1999) CR interventions manage to attenuate age-related increase in leptin levels, similar to our results.

Moreover, aging and many age-associated diseases have demonstrated a progressive increase in insulin levels in humans and rodents (Meigs et al., 2003). In addition, insulin works by

regulating peripheral glucose homeostasis and it is an important neuromodulator that contributes to neurobiological processes (Akintola & van Heemst, 2015). Preceding outcomes (Fontana et al., 2010) on monkeys and rodents demonstrated that CR reduces insulin concentration levels. Our findings did not support the results found in the literature. It should be taken into account that our animals were fasted for 24h before taking the blood sample, which may have affected the normal pattern of insulin secretion. It is also known that metabolic changes during overnight fasts lead to a decreased secretion of insulin (Cotero & Routh, 2009). Further research should consider taking multiples samples of insulin at different time points from the different groups of animals, instead of just one sample, in order to ensure a reliable comparison.

2.3.4 Insulin-like growth factor 1

Figure 51 depicts the plasma levels of IGF-1 (pg/mL). ANOVA showed differences between groups ($F_{(2,22)}$= 9.693, p= 0.001). Post Hoc analysis showed differences between the Young and CR animals (p = 0.006) and *Ad Libitum* group (p = 0.001). IGF-1 levels were higher in the young group of animals.

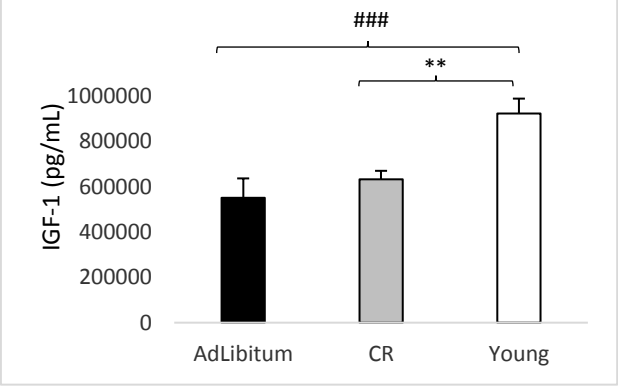


*Figure 51: Plasma levels of IGF-1 (pg/mL) in Ad Libitum, CR and Young group of animals. Values are mean ± SEM. ###p < 0.001 between Ad Libitum and Young group and **p < 0.01 between CR and Young group of animals.*

Partial discussion for plasma IGF-1 levels

IGF-1 is required for normal development of the body during the growth process. In general, high levels of IGF-1 have been shown to be protective against several age-related diseases such as type 2 diabetes, osteoporosis and cognitive decline (Barzilai & Bartke, 2009). Insulin/IGF like signaling is involved in the determination of lifespan and it has diverse functions. Therefore, mutations in this pathway can affect growth, development, stress resistance, fecundity and lifespan (Broughton & Partridge, 2009). This hormone is segregated during all lifespan, reaching a maximum peak during adulthood (human up to 30 years or rat 4-5 months) and enduring a decline in levels during aging (Yamamoto et al., 1991). Our results demonstrated an effect of age on this variable. Previous studies (Sonntag et al., 2005) found that long-term GH and IGF-1 replacement improves learning and memory in aged rats, supposedly via an increased neurogenesis, vascular density and alteration of the NMDA receptors (Fontana et al., 2016). The application of a CR diet could mimic the aging process in a stress-related response, which tends

to decrease levels of IGF-1 (Breese et al., 1991). In fact, previous findings about IGF-1 and CR depicted different outcomes. The dietary intervention can decrease (Fontana et al., 2008) or increase (Sonntag et al., 2005) IGF-1 levels, thus acting as a metabolic/hormonal key. Our findings suggest that aged animals (CR and *Ad Libitum* groups) presented the normal decreased levels of IGF-1 due to the aging process (Yamamoto et al., 1991). Therefore, the lowered levels of IGF-1 that our two aged group of rodents presented, act as a clear indicator that the individuals are in a state of "sensing" energy restriction (Fontana & Klein, 2007) as they are going through the aging process.

2.3.5 Corticosterone

Figure 52 depicts corticosterone levels (ng/mL) in blood plasma in the three groups of animals. ANOVA showed differences between groups ($F_{(2,22)}$ = 5.170, p = 0.014), and Post Hoc analysis revealed differences between CR and Young animals (p = 0.021) and a tendency towards significance (p = 0.054) between *Ad Libitum* and Young groups. Both groups of old rats showed higher levels of corticosterone than the Young animals.

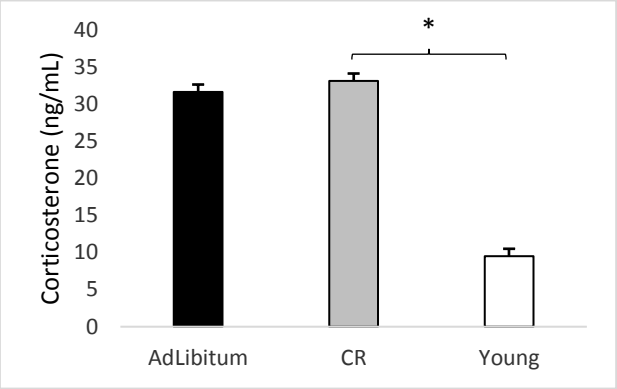


Figure 52: Plasma levels of corticosterone in ng/mL in Ad Libitum, CR and Young groups of animals. Values are mean ± SEM). *p < 0.05 between CR and Young groups.

Partial discussion for blood plasma corticosterone

Corticosterone and cortisol are hormones that are used as markers of "stress indicators" in many of the studies on rodents (Barzilai & Bartke, 2009). The aging process is accompanied by an upregulation of circulating levels of corticosterone in the blood (Montaron et al., 2006), which is in support of our results. Foregoing data (Moneo et al., 2017) found similar levels of corticosterone in *Ad Libitum* and CR aged animals, which confirms the lack of effect of CR in terms of stress. In addition, CR rats tend to present higher levels of blood corticosterone than their *Ad Libitum* counterparts due to the fasting pattern (Levay et al., 2010). However, our results did not show this increase. This could be partly explained by the social interaction facilitated by the paired-housed conditions of the present experiment. This situation has been proven to lower corticosteroid levels in rats, which does not happen in animals that were lonely-housed (Claassen, 1994).

2.4 Correlations

2.4.1 Correlation between monoaminergic transmission and behavioral data

In order to study the possible relations between the monoaminergic system in the HPC and cognitive processes that comprise the memory tasks that animals carried out, we performed correlations between NA, DA, 5-HT, their rate-limiting precursor TH and TPH, as well as the metabolites DOPAC and 5-HIAA, and the behavioral data from the ODT and MWM. The aim of this analysis was to evaluate whether variations of the monoaminergic system underlies at least in part, the age-related cognitive decline observed in old animals. Moreover, we also assessed whether CR is able to reduce the alterations observed in the monoaminergic system, which could help to explain the reduction in age-related memory impairment.

Table 17 shows the main significant correlations that have been found between the dopaminergic or serotonergic neurotransmitters or metabolites, which is indicated in the row and in the column the behavioral task. Significant correlations were detected between TPH, TH, 5-HIAA and DA and the test phase of the MWM, specifically in the variable time at the target quadrant and target annulus. Moreover, no significant correlations were found between these variables and the ODT performance or in the learning phase of the MWM.

Table 17: Summary of the significant correlations between the monoamines levels in the HPC (TPH, 5-HIAA, TH) FC (DA, DOPAC) and variable time at the platform quadrant of the MWM P and r indicated the levels of significance.

	MWM
	Test
TPH	HPC r= 0.467 P= 0.034
5-HIAA	HPC r= 0.612 P= 0.018
TH	HPC r= 0.446 P= 0.048
DA	FC r= 0.618 P= 0.014
DOPAC	FC r= 0.525 P= 0.044

Serotoninergic system and long-term memory evaluated in the MWM test

The correlational analysis between the hippocampal serotonin and spatial memory showed that both the amino acid TPH and the metabolite 5-HIAA positively correlated with the performance in the MWM test. Specifically, analysis via Spearman indicated a significant positive correlation showing that animals with higher levels of TPH (r= 0.467, p= 0.034) and 5-HIAA (r= 0.621, p= 0.018) in the HPC, also spent more time in the target annulus and/or the target quadrant during the test phase of MWM (Figure 53a and 53b). A better long-term spatial memory recall was assessed in the time spent in the target annulus or the target quadrant during the test phase of MWM.

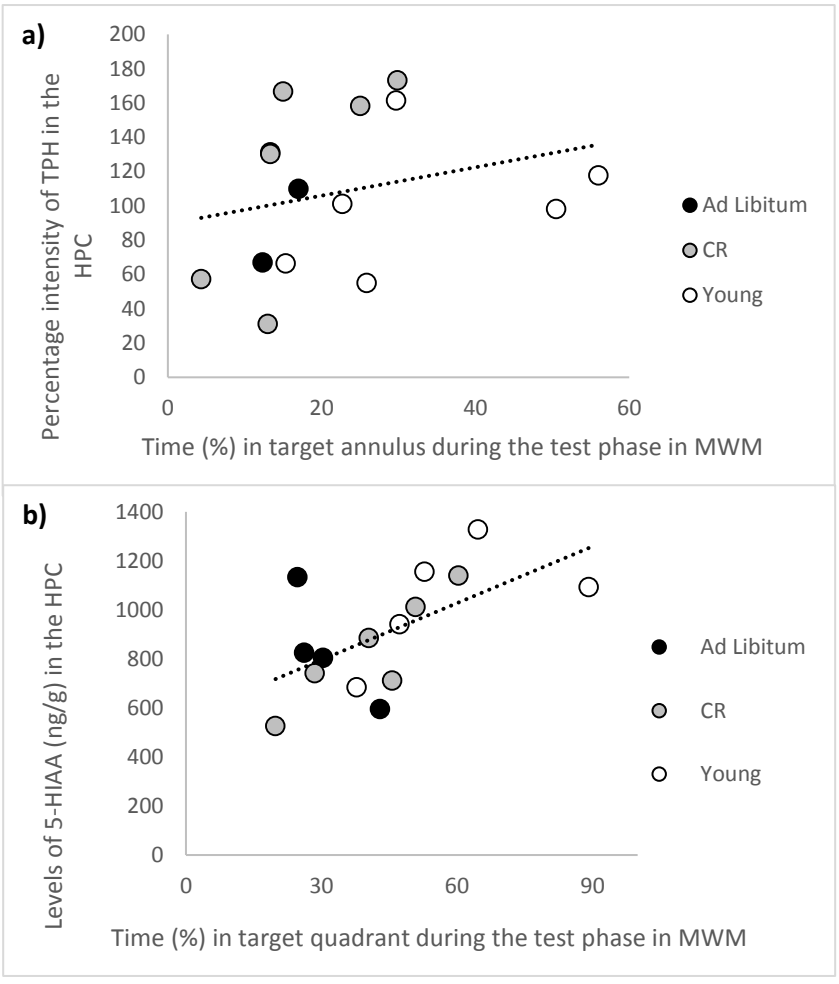


Figure 53: a) Spearman correlation analysis between percentage intensity of TPH in the HPC and time in target annulus in the MWM test b) Spearman correlation analysis between levels of 5-HIAA (ng/g) in the HPC in the target quadrant during the test phase in MWM.

Dopaminergic system and long-term memory evaluated in the MWM test

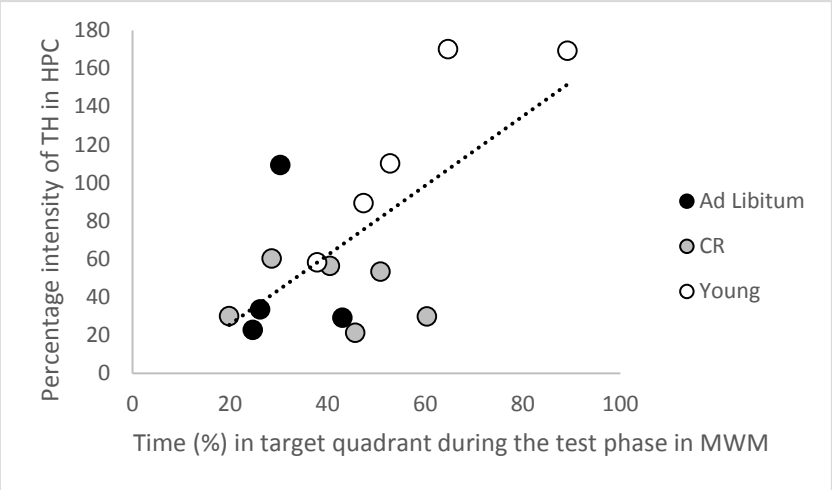
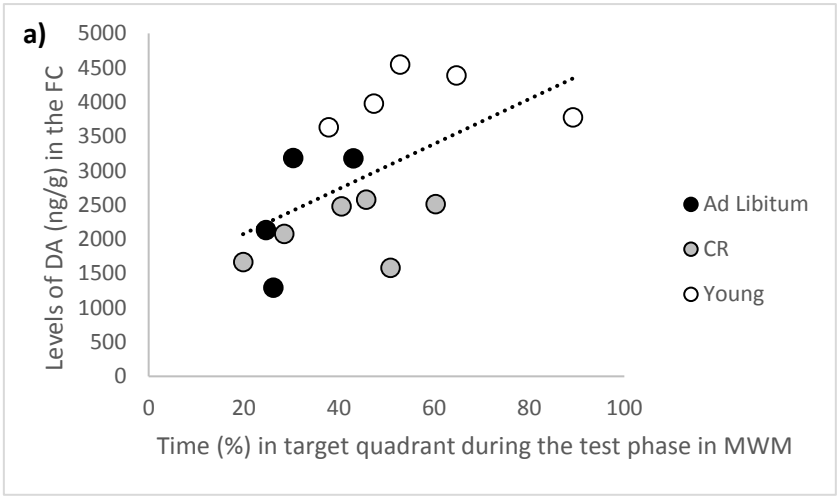


Figure 54: Spearman correlation analysis between TH in HPC and the time (in percentage) in the target annulus during the test phase in MWM.

Figure 54 shows the positive correlation (r=0.446, p=0.048) between Western Blot results regarding the percentage of TH intensity in the HPC, the rate-limiting precursor of DA, and the percentage of time spent in the target quadrant during the test phase of MWM. This analysis confirmed that subjects with a higher expression of TH in the HPC had better spatial long-term memory retention.

The correlational analysis between the dopaminergic system and the spatial memory showed that both NT DA (Figure 55a) and metabolite DOPAC (Figure 55b) in the FC were related to the time spent at the target quadrant during the test phase of MWM. Results showed a positive significant correlation in DA (r= 0.618, p= 0.014) and DOPAC (r= 0.525, p= 0.044), indicating that the more expression of DA and its metabolite DOPAC, the better long-term memory retention in the MWM.



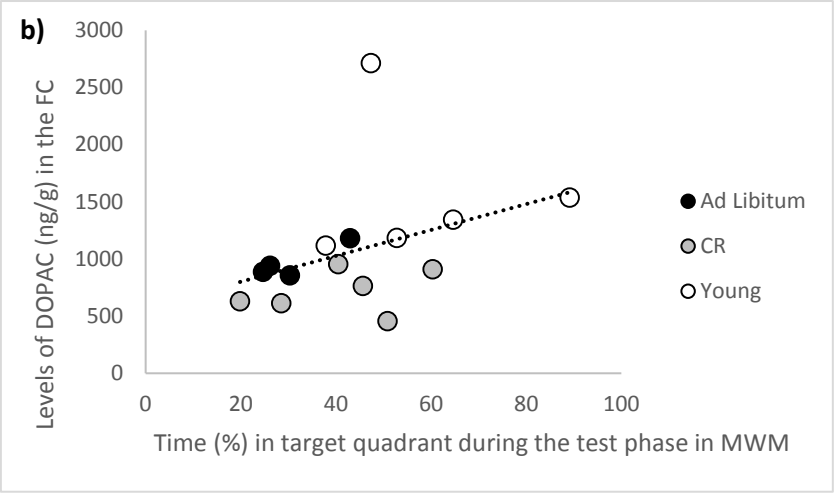


Figure 55: Spearman correlation analysis of both levels of DA (ng/g) in FC (a) and DOPAC in FC (b), between the NT and the time (in percentage) in the target quadrant during the test phase in MWM.

Partial discussion for the correlation between monoaminergic system and behavioral data

Present results demonstrate that levels of the serotoninergic precursor TPH, as well as levels of the metabolite 5-HIAA correlated with the long-term spatial memory retention in the MWM. In relation to TPH levels and spatial memory performance, no previous bibliography was found. However, previous results (Table 1) showed that there might be changes in the 5-HIAA levels in the HPC during aging. In that regard, and in agreement with our results, it has also been shown that HPC serotoninergic levels are lower in aged rats compared to young animals (Esteban et al., 2010). In fact, it has been suggested (Kemp & Manahan-Vaughan, 2008) that 5-HT receptors strongly influence the precise information processing in the HPC. These data agree with results coming from previous research (González-Burgos & Feria-Velasco, 2008; Kemp & Manahan-Vaughan, 2008), which have proposed that memory processes are partially regulated by neuromodulators such as 5-HT and DA. Nevertheless, several authors (Godefroy et al., 1987; Lee et al., 1994; Sirviö et al., 1994) have found higher levels of 5-HIAA in the HPC of aged rats, while other studies (Koprowska et al., 2004; Luine et al., 1990; Ponzio et al., 1982; Stemmelin et al., 2000) did not find differences in the levels of this metabolite in aged animals compared to adults. Moreover, our results revealed that the CR group presented a stronger correlation between serotoninergic levels and performance in the MWM, since it seems that the general distribution of the dot cloud of the CR group is better adjusted to the regression line. In contrast, correlations in the *Ad Libitum* and Young animals presented a greater dispersion, which may be related with intragroup differences in the cognitive performance.

Regarding the dopaminergic system, levels of TH in the HPC, and DA and DOPAC in the FC correlated with the test phase of MWM, confirming the relevance of this system in the formation

of spatial memory. Our results agree with previous ones (Mizoguchi et al., 2000) which demonstrated that chronic stress in rats might alter the dopaminergic function in the PFC, thus impairing memory. It has been proposed (Bubser & Schmidt, 1990) that DA in the PFC may function by suppressing the contextual interference during the delay period of cognitive tasks. This suggestion might help explain our results, since both aged group presented less levels of DA and DOPAC in the FC than young animals (Figure 44). However, the correlation between FC DA expression and the test phase of the MWM demonstrated a well-defined scatter dot cloud in the three groups of animals, demonstrating no intragroup differences. Young animals performed better, followed by CR animals. Both groups showed preserved long-term memory, whereas *Ad Libitum* animals did not. Previous studies (Frey et al., 1990) have pointed to a specific role of DA in hippocampal LTP temporal persistence. In this regard, the DA receptors D1 and D5 seem to be involved in the changes observed in terms of the synaptic strength associated to memory and LTP (O'Carroll et al., 2006). Altogether, the evidence presented above strengthens the link between DA and hippocampal memory (Moraga-Amaro et al., 2016), and supports the correlations found in this study.

2.4.2 Correlation between glutamatergic system and behavioral data

In order to study the possible relations between the glutamatergic system in the HPC and the FC and cognition, we performed correlations between AMPA1, AMPA2 and NMDAR2A subunits and the behavioral data obtained from the ODT and MWM. The aim of this analysis was to evaluate whether variations of the glutamatergic system underlies, at least in part, the age-related cognitive decline observed in old rats. Moreover, we also assessed whether CR is able to reduce the alterations observed in this NT system.

Table 18 shows the Spearman correlations between the glutamatergic subunits in the HPC and FC and the behavioral data. The receptors subunits of NMDAr and AMPAr are displayed in the rows, while the performance variables for the acquisition and test phases of ODT and MWM are depicted in each column. The learning process in the ODT was analyzed using the mean latency to find the reinforcement during the acquisition, whereas the MWM performance was assessed using the time it took the animals to find the platform, as well as the time in the target quadrant in the last session of the acquisition phase. Long-term memory in ODT was analyzed using the latency to find the reinforcement, while the MWM performance was assessed using the time

animals spent in the target annulus or in the quadrant during the test. The results exhibited the significant correlation or the tendency towards significance between the following variables.

Table 18: Summary of the correlations between the glutamatergic receptors subunits and the behavioral performance in ODT and MWM. For each correlation, the receptor subunit, the brain area and the phase of the behavioral task are indicated. Significant or tendency to be significant of P and r are depicted for each correlation.

	ODT		**MWM**	
	Acquisition	**Test**	**Acquisition**	**Test**
AMPA1	FC r= -0.489 P= 0.032	HPC r= -0.707 P= 0.003	HPC r= -0.0528 P= 0.032	HPC r= 0.47 P= 0.052
AMPA2				HPC r= 0.577 P= 0.019
NMDAR2A	FC r= -0.456 P= 0.059		FC r= 0.588 P= 0.017	

AMPA1

Figure 56 depicts the correlation between the intensity of AMPA1 subunit in FC and the mean latency to find the reinforcement during the acquisition in the ODT phase. Analysis via Spearman correlation indicated a significant negative correlation between these the variables (r= -0.489, p= 0.032), showing that the higher the expression of AMPA1 subunit in FC, the better the performance during the ODT acquisition.

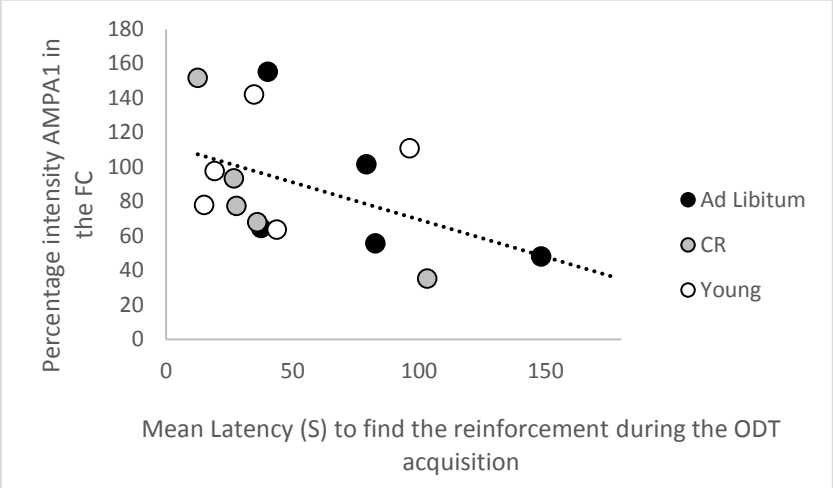


Figure 56: Spearman correlation analysis between AMPA1 subunit in the FC and the mean latency (s) to find the reinforcement during the ODT acquisition.

Figure 57 shows the correlation between the intensity of AMPA1 subunit in HPC and the mean latency to find the reinforcement during the test session of the ODT task.

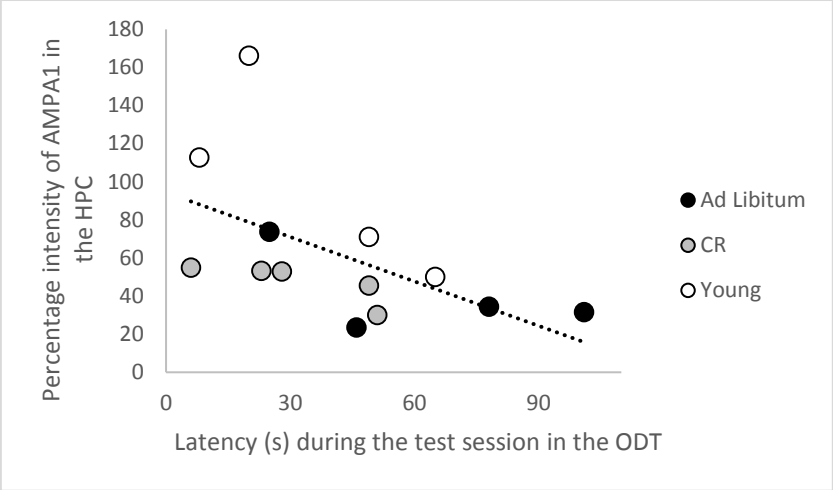


Figure 57: Spearman correlation analysis between AMPA1 in the HPC and latency (in seconds) during the test session in the ODT phase.

Analysis via Spearman indicated a negative significant correlation (r= -0.707, p= 0.003), indicating that the more expression of AMPA1 subunit in HPC the better memory retention.

Figure 58 represents the correlation between the intensity of AMPA1 subunit in HPC and the mean latency to find the platform in the last (5th day) acquisition session in MWM.

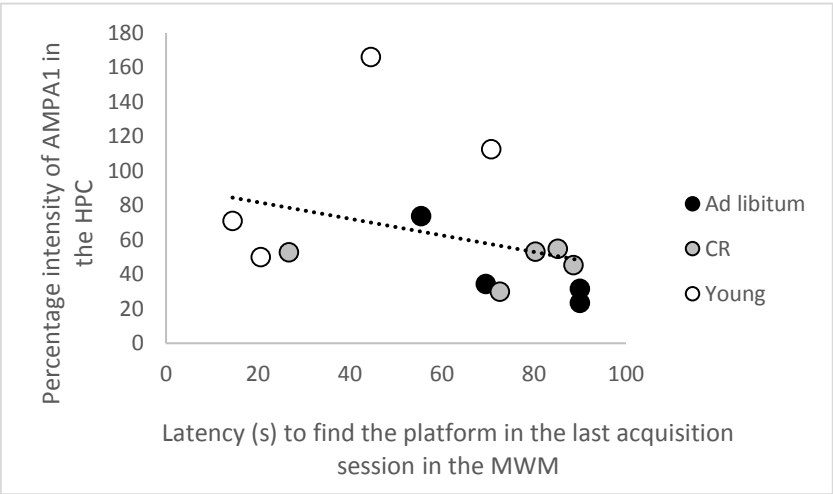


Figure 58: Spearman correlation analysis between AMPA1 in HPC and the latency (in seconds) to find the platform in the last acquisition session in the MWM.

Spearman analysis indicated a significant negative correlation (r= -0.528, p= 0.032) between both variables. Subjects with higher expression of AMPA1 subunits in HPC presented lower latencies to find the platform, suggesting a better learning.

Figure 59 demonstrated a correlation between the expression of AMPA1 subunit in HPC with the mean time spent in the target annulus during the test phase of MWM.

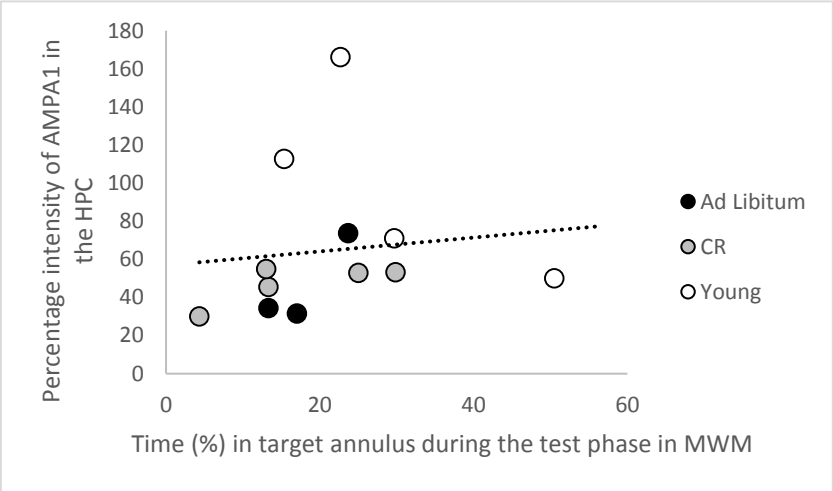


Figure 59: Spearman correlation analysis between AMPA1subunit in HPC and the time (in percentage) in the target annulus during the test phase in MWM.

Analysis via Spearman indicated that this correlation showed a tendency towards significance (r= 0.470, p = 0.052), showing that subjects with higher levels of AMPA1 subunits in HPC showed better long-term memory retention.

AMPA2

Figure 60 shows the correlation between Intensity of AMPA2 subunit in HPC and the time spent at the target quadrant during the test phase of MWM. An analysis via Spearman indicated a significant positive correlation (r= 0.577, p= 0.019) between Intensity of AMPA2 subunit in HPC and the time spent at the target quadrant during the test phase of MWM, demonstrating that subjects with higher levels of AMPA2 in the HPC showed better long-term memory.

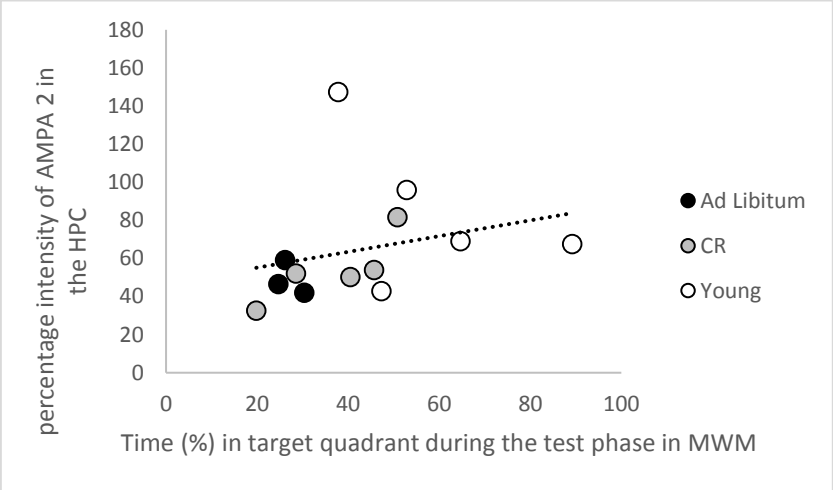


Figure 60: Spearman correlation analysis between AMPA2 subunit in HPC and the time (in percentage) in the target quadrant during the test phase in MWM.

NMDAR2A

The figure 61 depicts the correlation between the intensity of NMDAR2A subunits in FC with the mean latency to find the reinforcement during the acquisition in the ODT. Analysis via Spearman indicated a negative correlation with tendency towards significance (r= -0.456, p = 0.059), indicating that the higher expression of NMDAR2A subunit in the FC, the lower latency to find the reinforcement.

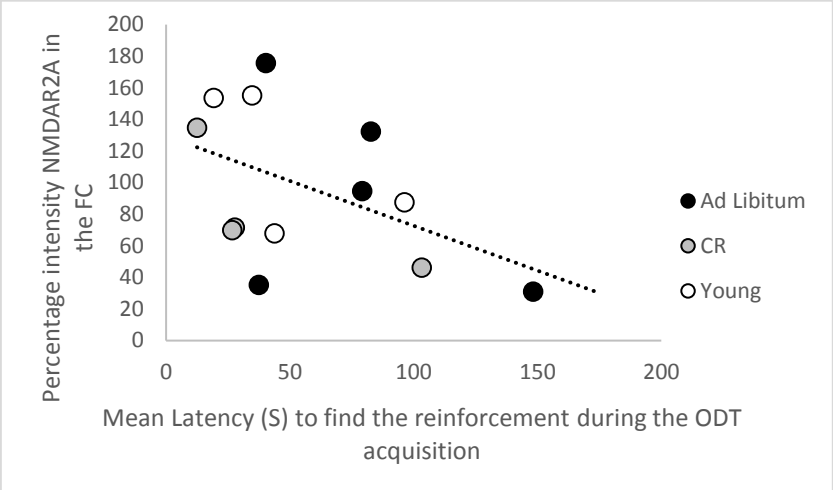


Figure 61: Spearman correlation analysis between NMDAR2A subunit in the FC and the mean latency (s) to find the reinforcement during the ODT acquisition.

Figure 62 illustrates the correlation between the intensity of NMDAR2A in FC and the time in the platform quadrant during the last (5th day) acquisition session in MWM. Analysis via Spearman indicated a significant positive correlation (r= 0.588, p= 0.017), since subjects with higher expression of this receptor in the FC showed a better performance in the acquisition session.

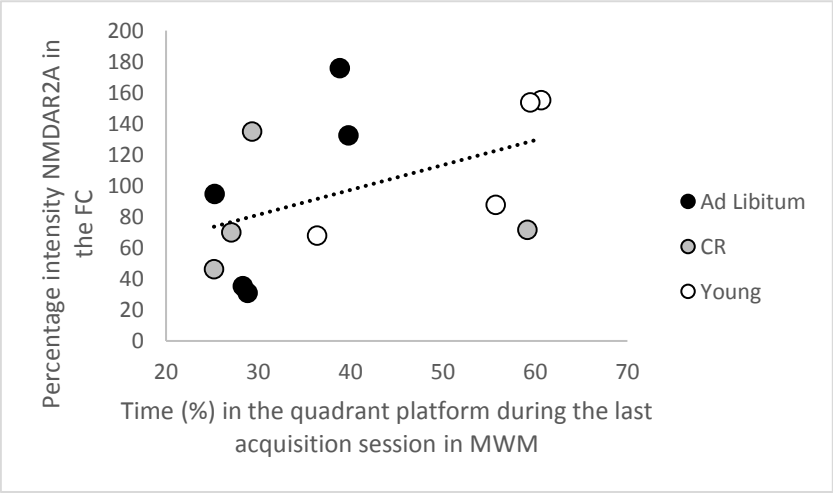


Figure 62: Spearman correlation analysis between NMDAR2A in the FC and the time (In percentage) in the platform quadrant during the last session in MWM.

Partial discussion on the correlations between glutamatergic receptors subunits and behavioral data

Our results indicated a strong correlation between hippocampal AMPA1 and AMPA2 receptors and several variables in the MWM and ODT memory tests. Thus, FC AMPA1r subunit strongly correlated with the acquisition phase of the ODT, while levels of these receptors in the HPC correlated with test variables of the ODT and the MWM. Previous studies have found that CR intervention managed to maintain similar levels of AMPA1 and 2 subunits in the HPC of aged animals compared to young animals; however, both receptors seemed to be downregulated in *Ad Libitum* animals (Adams et al., 2008; Shi et al., 2007). Moreover, outcomes from the present study agree with results coming from previous research (Liang et al., 1994), which demonstrate a correlation between NMDAr in the FC and the MWM. This confirms the implication of those receptors in learning and memory processes. In general, a lower dispersion cloud was found in the CR group compared to *Ad Libitum* animals, which could be related to a similar execution, meaning that the CR animals did not show intragroup differences. In contrast, the *Ad Libitum* group presented a higher dispersion, and therefore, an unequal execution in cognitive tasks.

Our results agree with evidence coming from genetic and pharmacological studies (Liang et al., 1994; Kullmann et al., 2000; Riedel et al., 2003) that have demonstrated that both AMPA and NMDA ionotropic glutamate receptor are involved in hippocampal-dependent tasks, such as MWM, as well as in an FC dependent task, such as ODT (Tronel, 2002). Moreover, AMPA1 subunit has been shown to be necessary for synaptic plasticity (Kullmann et al., 2000), while its phosphorylation is required for LTP expression and memory retention (Lee et al., 2003). This confirms the relation between AMPA1r subunit and learning and memory processes.

2.4.3 Correlation of blood plasma levels of hormones and proteins and behavioral data

In order to study the possible relations between the plasma levels of hormones, lipids and other proteins and cognition, we performed correlations between corticosterone, triglycerides, glucose and IGF-1 and the behavioral data obtained from the ODT and MWM. The aim of this analysis was to evaluate whether variations of blood plasma levels of these parameters underlies, at least in part, the age-related cognitive decline observed in old rats. Moreover, we also assessed whether CR is able to reduce the alterations in the levels of plasma hormones and proteins observed in aged animals.

Table 19 shows the correlations between the results of the blood plasma analysis and the behavioral data. Plasma levels of hormones and lipids are depicted in the rows, whereas the behavioral data is shown in each column. Learning in the MWM was assessed by measuring the

time the animals spent in the target quadrant in the last session of the acquisition phase. Memory was evaluated as the time they spent in the target quadrant during the test phase. Results showed significant correlations between the variables.

Table 19: Summary of significant correlations between blood plasma results and behavioral data.

	MWM	
	Acquisition	Test
Corticosterone	r= -0.721 P= 0.001	
Triglycerides	r= -0.578 P= 0.015	
Glucose		r= -0.521 P= 0.046
IGF-1		r= 0.604 P= 0.017

Corticosterone

Figure 63 depicts the correlation between the levels of corticosterone and the time in the platform quadrant during the last acquisition session in the MWM. Spearman analysis demonstrated a significant negative correlation (r= -0.721, p = 0.001) indicating that higher levels of corticosterone were related to a worse MWM acquisition.

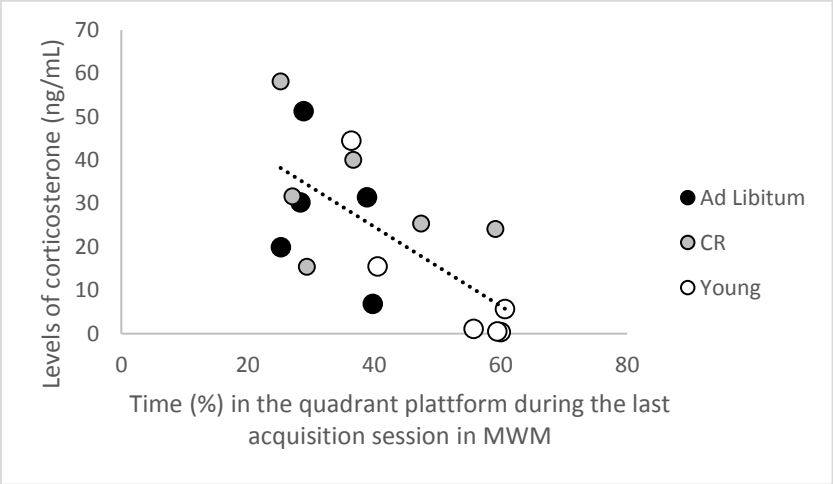


Figure 63: Spearman correlation between levels of corticosterone (ng/mL) and the time (in percentage) in the quadrant platform during the last acquisition session in MWM.

Triglycerides

Figure 64 depicts the correlation between the levels of triglycerides and the time in percentage in the platform quadrant during the last acquisition session in MWM.

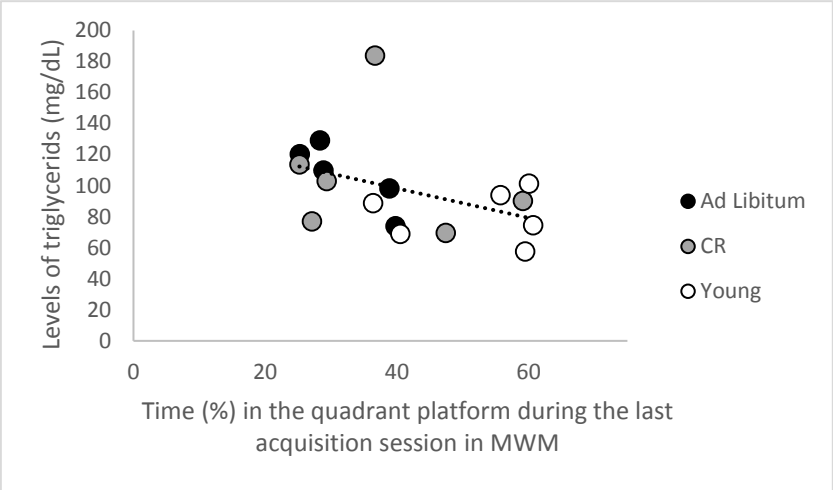


Figure 64: Spearman correlation analysis between levels of triglycerides and time in the platform quadrant during the last acquisition session in MWM.

The analysis via Spearman correlation indicated a significant negative correlation (r= -0.578, p = 0.015), showing that subjects with higher triglycerides had a worse performance in the test.

Glucose

Figure 65 shows the correlation between the level of glucose and the time spent in the platform quadrant during the test phase of MWM.

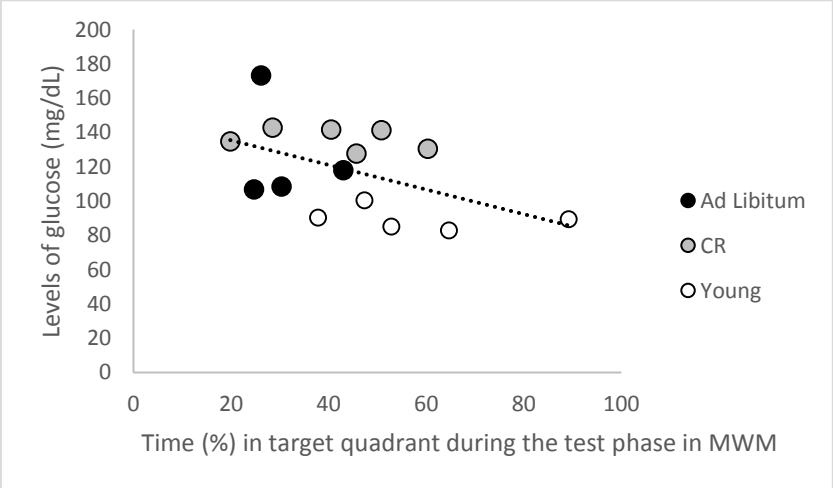


Figure 65: Spearman correlation analysis between levels of glucose (mg/dL) and the time (in percentage) in the target quadrant during the test phase in MWM.

Analysis via Spearman indicated a significant negative correlation (r= -0.521, p = 0.046). Thus, subjects with higher levels of glucose showed a worse long-term memory.

Insulin-growth factor 1

The correlation between the levels of IGF-1 and the time in the target quadrant during the test phase in MWM was analyzed via Spearman. Results indicated a direct and significant correlation (r= 0.604, p = 0.017), in which subjects with higher levels of IGF-1 presented a better long-term memory as shown in Figure 66.

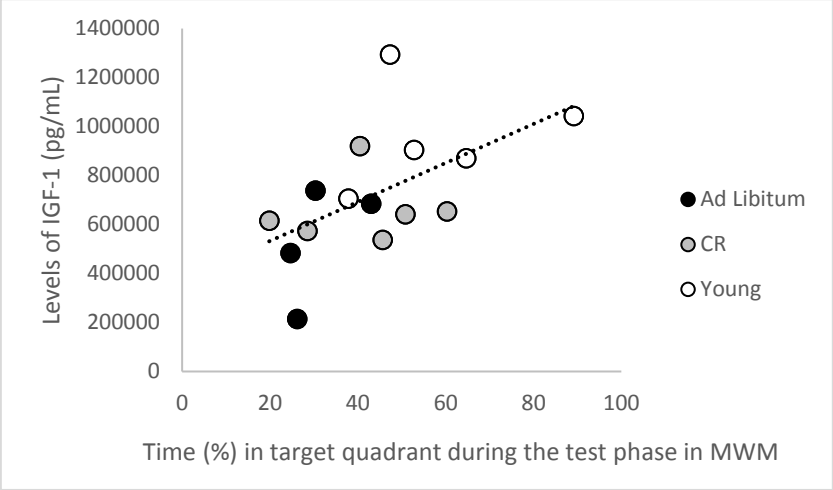


Figure 66: Spearman correlation analysis between levels of IGF-1 (pg/mL) and the time (in percentage) in the target quadrant during the test phase in MWM.

Partial discussion on blood plasma levels of hormones, lipids and proteins and behavioral data

Our results indicated that both aged groups had higher levels of corticosterone than the young rats, confirming an age-depended increase of this hormone (Montaron et al., 2006). In fact, the scatter cloud of points in the correlation is well defined between groups, which could involve an affectation in each subsample of subjects at a general level. This could imply that memory could be suffering from interference in aged animals due to higher levels of corticosterone. Previous research carried out on aged Long Evans rats (Bodnoff et al., 1995) demonstrated that long-term exposure to elevated corticosterone levels can result in spatial learning deficits in the MWM, corroborating our results. In addition, it has been previously described (Sapolsky et al., 1985) that prolonged treatment with high corticosterone levels can produce changes in the HPC, reducing the number of neurons and accelerating the aging process. In addition, repeated stress, which causes higher corticosterone levels, triggers an impairment during the acquisition of the eight-arm radial maze in rats (Luine et al., 1994). However, the behavioral control tests in the OF and the EPM did not demonstrated significant differences between animals in their anxiety levels, which leads us to think that higher corticosterone levels are a consequence of the aging process itself. Aging is indeed well-known for being a stressful phenomenon (Breese et al., 1991).

Regarding the correlation between triglycerides and the learning phase of the MWM, no information about the effects of triglycerides on cognitive processes in rodents has been reported. Therefore, our results will be discussed in the context of human studies. Prior

experiments (Morley & Banks, 2010) have demonstrated that high cholesterol and triglycerides levels have been implicated in age-related cognitive decline and pathologies as Alzheimer's disease or vascular dementia. High blood plasma triglycerides have been especially associated to general cognitive decline and worse semantic memory in non-demented aged humans from 55 to 80 years old (de Frias et al., 2007; Morley & Banks, 2010). In addition, both age and triglycerides were linked to deficits in a comprehensive neuropsychological examination task in humans (Leritz et al., 2016). Moreover, another study (Vinkers et al., 2005) on aged human population, demonstrated that humans with previous cardiovascular pathologies, which are usually related to higher triglycerides, presented a worse memory performance. Thus, higher triglycerides levels may contribute to cognitive decline in old age. In general, human findings could provide evidence to support the link between high serum triglycerides and a detriment in MWM acquisition found in the present experiment, although more research in rodents is needed.

Regarding the glucose correlation, the results from the present study showed that both aged groups presented higher levels compared to young control animals, indicating an age impairment in those subjects. This detrimental effect was reverted by the CR diet. It is well known that acute glucose intake can enhance learning, memory and attentional processes in rodents and humans (Korol & Gold, 1998). However, it has been suggested that constant hyperglycemia and insulin resistance can produce hippocampal damage and deficits in cognitive behavior (Valladolid-Acebes et al., 2011). Moreover, a study (Malone et al., 2008) on young Wistar rats, demonstrated a link between hyperglycemia and adverse effects on neuronal structural changes, as well as impaired long-term hippocampal spatial memory in the radial-arm water maze. Interestingly, maternal hyperglycemia in Sprague-Dawley rats leaded to deficits in learning and memory in their offspring (Kinney et al., 2003). All this information tends to support our results in which subjects which higher levels of glucose seem to present a worse hippocampal long-term memory.

Finally, our results demonstrated that lower levels of IGF-1 could be related to worse long-term memory retention. IGF-1 is a neuroprotective hormone which is decreased during aging (Yamamoto et al., 1991). Prior studies (Lupien et al., 2003) have indicated that brain IGF-1 is essential for learning and memory in the MWM and, therefore, the loss of this protein may contribute to cognitive impairments in adult rats. In mice, the inactivation of the production of IGF-1 in the liver of 15 to 18-month-old mice, as a results of an 80 to 85% reduction of circulating plasma IGF-1 levels, showed an impairment in spatial learning and memory in the MWM (Svensson et al., 2006).

VI. GENERAL DISCUSSION

VI. GENERAL DISCUSSION

The progressive aging of the population and the consequent deterioration of health and cognitive functions in the elderly has caused the need to investigate the physiological mechanisms involved in the aging process. This knowledge will be necessary to design new procedures to ensure a healthy senescence and reduce the onset of neurodegenerative diseases. Consequently, the main objective of this research was to study whether dietary intervention consisting of reducing caloric intake throughout life could be an effective method for combating age-related cognitive decline. For this purpose, we have investigated the effects of CR on short and long-term memory in aged animals. In addition, to understand the physiological mechanisms underlying the possible behavioral benefits of this type of dietary intervention, several biochemical variables have been analyzed. These include the levels of cerebral monoamines, DA, 5-HT, NA, precursors and metabolites, glutamatergic receptors NMDA and AMPA, the presynaptic marker SYP and different blood hormones such as corticosterone, insulin, leptin, IGF-1, and other plasma parameters related to healthy metabolism like triglycerides, glucose, HDL, LDL, albumin, calcium or ALP.

CR improves health and wellbeing in old rats

Our research has demonstrated that CR can improve the general health of aged animals. This may be due to the fact that CR enhanced some blood plasma parameters that might be altered due to aging. For example, albumin, a protein related to the regulation of oncotic pressure of blood by some proteins, was higher in the CR group of old rats compared to *Ad Libitum* and Young groups. In addition, ALP was also increased in the CR old group compared to *Ad Libitum* animals, indicating no malnutrition or anemia in the CR intervention group. In addition, levels of cholesterol, a sterol related to animal cell structure that serves as a precursor for the synthesis of steroids hormones and vitamins (Hanukoglu, 1992), did not differ between young and old groups of rats in spite of the age and the dietary intervention. Nevertheless, other variable related to health, such as glucose plasma levels were increased in both groups of old rats, which is consistent with the typical expected symptoms of aging. Furthermore, CR was not able to reverse this alteration as previously demonstrated (Sonntag et al., 2005). Nonetheless, other variables such as triglycerides and calcium need further investigation, since our results are not conclusive enough to affirm whether or not CR restores the blood plasma levels of these parameters to the levels observed in young rats.

As previously mentioned in the introduction section, the pattern of insulin/IGF-1 levels changes during aging. IGF-1 is produced over the course of an animal's life reaching a peak during

adulthood with levels then declining during aging (Yamamoto et al., 1991). Both groups of older animals in our experiment showed decreased levels of IGF-1 compared to young controls, indicating that the individuals were in a state of "sensing" energy restriction (Fontana & Klein, 2007) and going through the aging process. However, no changes in insulin plasma levels between groups were found. It has been suggested (Ma et al., 2017) that part of the beneficial effects of CR on health could be due to the regulation of insulin/IGF-1 levels, which decrease moderately with the dietary intervention, but our outcomes cannot clearly demonstrate that assertion due to the fact that similar levels of insulin were observed between groups. In addition, leptin, a hormone that is a good predictor of global adiposity status (Weigle et al., 1997), decreased in CR old rats, indicating that the dietary intervention was able to reverse the enhancement previous investigations demonstrated (Imbeault et al., 2004; Wisse et al., 1999). Lower levels of leptin are associated with protection against cardiovascular risk and mortality (Golbidi et al., 2017). Furthermore, our results showed an age effect in blood plasma corticosterone levels, in agreement with previous studies (Moneo et al., 2017). Moreover, our results demonstrated that CR diet tended to increase the level of corticosterone in blood plasma in old rats, in agreement with previous results (Levay et al., 2010). In general, we might conclude that all animals were in good shape and condition during the performance of the behavioral tasks, as demonstrated by the aforementioned biochemical parameters as CR intervention never worsened the plasma levels of any of the parameters analyzed. In fact, it improved some of them or kept them at adequate levels for the aging process itself.

CR does not alter motor activity and anxiety levels in old rats

Our results demonstrated that CR aged rats exhibited slightly less locomotor activity when compared to young animals, as assessed in the OF and EPM. However, prior studies on the locomotor activity of aged animals is controversial and non-conclusive when it comes to the effects of CR. One study suggests that the dietary intervention might increase physical activity (Geng et al., 2007), whereas another argues the opposite (Kuhla et al., 2013). The different outcomes lead us to consider the possibility that CR affects locomotor activity in different ways. For instance, as previously explained (Results 1.2), the time of day when the animals were fed might affect the activity and the irritability of the rodents (Smith & Metz, 2005). Furthermore, as suggested (Levay et al., 2007) the slower motor activity of the CR animals may merely reflect food seeking behavior, as the animals stop exploring to look for food. However, in our experiment there were no differences between groups in the swim speed in the acquisition phase of the MWM, which lead us to conclude that when the task lasts longer all animals

presented similar motor activity patterns and the time of food consumption did not affect the task.

Moreover, CR did not increase anxiety levels in older animals as the behavioral performance in the OF, the EPM and the *thigmotaxis* in the MWM did not show differences between groups. However, levels of blood plasma corticosterone were elevated in both aged groups, following an expected age-related pattern (Moneo et al., 2017), which may have affected the anxiety-like behavior, but it was not observed in our results. For these reasons, we can conclude that the dietary intervention did not affect the anxiety level and locomotor activity as previous studies demonstrated (Jahng et al., 2007; Kuhla et al., 2013).

CR attenuates brain aging

As mentioned previously, the benefits of CR are not restricted exclusively to peripheral health variables, such as the plasma levels of certain hormones, but also to changes in brain biochemistry. In this sense, our results seemed to confirm that CR might modify monoaminergic and glutamatergic neurotransmission, the main biochemical systems that are impaired during aging (Ponzio et al., 1982). On the one hand, our data demonstrated that CR improved dopaminergic transmission in the striatum and noradrenergic transmission in the HPC and FC, which is affected during aging. On the other hand, levels of DA and metabolites decreased in both aged groups in the FC, as previously described (Eppinger et al., 2011; Koprowska et al., 2004; Lee et al., 1994; Míguez et al., 1999; Ponzio et al., 1982). In addition, CR animals also presented similar levels to the Young animals in most of the serotoninergic related precursors and metabolites in the HPC, while they showed significant differences from the old *Ad Libitum* group. These results confirm the hypothesis that CR might negate the age-related decrease in brain monoamines (Ponzio et al., 1982). Furthermore, our results showed stable and similar levels of TPH in the FC and the HPC in the three groups of animals, demonstrating no aging or CR effects on them. When it comes to the levels of the serotoninergic NTs, our results verified that aged animals showed a general reduction of 5-HT in the HPC, occipital cortex and FC, effects which were reversed by CR, as previously observed (Sirviö et al., 1994, Stemmelin et al., 2000). In addition, 5-HIAA in the HPC was also lower in *Ad Libitum* animals but not in CR group, in agreement with previous reports (Esteban et al., 2010). Finally, in relation to NA levels of NT, no differences between groups were found in all the areas studied, with the exception of the HPC, in which CR animals presented higher levels than both *Ad Libitum* and Young rats. Therefore, we can conclude that in general, but with some exceptions, CR seems to reverse the age-related loss of monoamines. Further studies are needed to confirm this suggestion.

In addition, our results also showed that glutamatergic AMPAr subunits levels were decreased in *Ad Libitum* animals compared to Young and CR groups in the HPC, as previously demonstrated in AMPA1 (Adams et al., 2008; Newton et al., 2008; Shi et al., 2007) and in AMPA2 subunits (Adams et al., 2008; Shi et al., 2007). In both cases, CR reversed the age-related loss of these AMPAr subunits, as previously reported (Adams et al., 2008). However, no effects of CR on those receptors seem to have been found in the FC, as all the groups maintained stable levels. Regarding NMDAr subunits NMDAR1 and NMDAR2A levels, our data recorded no differences between groups in FC and HPC. Previous studies (Eckles-Smith et al., 2000) detected a decrease of these subunits in old rats that was attenuated by CR (Adams et al., 2008; Monti et al., 2004; Newton et al., 2008; Shi et al., 2007), although other investigations (Monti et al., 2004) also failed to prove a beneficial effect of CR on these subunits. In general, NMDAr seemed to stay well-regulated during aging in this experiment and the application of a 30% CR did not modify the levels of those receptors.

Finally, no differences were detected between groups in relation to the presynaptic protein SYP. Previous studies (Adams et al., 2008; Singh et al., 2015) into the effects of CR on SYP indicated that CR may increase the expression of this protein in the HPC and the HT and that hippocampal SYP is decreased in old *Ad Libitum* rats (Portero-Tresserra et al., 2018). Nevertheless, our experiment was unable to confirming these results. Therefore, our data suggests that a 30% CR diet did not affect SYP levels in the HPC, the striatum or the FC, demonstrating that CR did not change the presynaptic membrane protein which plays a role in neurotransmission in hippocampal neurons (Weimer & Jorgensen, 2003) and synaptic plasticity (Hajjar et al., 2013).

CR improves short and long-term memory

As mentioned above, the main objective of this study was to determine whether the lifelong CR might be capable of negating age-induced short-term and long-term memory impairment in memory tasks. The principal results of this research have demonstrated that the use of CR in old rats from the age of 4-months, attenuated age-related memory decline in short and long-term memory, as the performance of the old CR rats in the object recognition task in the Y maze and the MWM test was better than that of the *Ad Libitum* group and did not differ from the Young group.

Short-term memory was assessed through object recognition in the Y maze. The results of our experiment demonstrate that the CR old rats and Young group showed better retention when compared to the *Ad Libitum* animals. Previous investigations (Shukitt-Hale et al., 2001) demonstrated that the age process *per se* impaired spatial representation and the ability to

detect a change in the environment. In fact, some authors (Bevins & Bardo, 1999) have suggested that a decreased interaction with the novel object can be indicative of anhedonia. Therefore, place and object recognition might be suitable procedures for analyzing the neural processes underlying the affinity effects for the object or place. It is also supposed that widespread neural networks may be required to facilitate this process (Richardson et al., 2011). Such networks seem to be affected during aging (Reuter-Lorenz, 2002), which may impair novel object recognition in aged animals. All these reasons lead us to conclude that CR ameliorated the effects of aging in the novel object recognition task. Therefore, the use of this pioneering task to analyze the effects of dietary interventions such as long-term CR on aged animals has proved to be a good instrument since it avoids the use of food-rewards and fasting that can lead to biased results. In general, the results of this task show that aged animals on a CR diet can maintain levels of spatial representation and recognition of objects equivalent to that of young animals. In contrast, as expected, the *Ad Libitum* group showed an impairment thus demonstrating the effects of aging on the recognition of objects (Dellu et al., 1992).

Regarding long-term memory, we investigated the effects of CR in the ODT and MWM. On the one hand, results from the ODT indicated that a CR diet did not enhance the performance of the aged animals in this olfactory food-reinforced task with an intertrial time of 72h between the acquisition and the test phase. In fact, no differences in performance were detected between groups, although *Ad Libitum* animals showed a slightly poorer memory than the other two groups. The lack of clear effects on ODT, a food rewarded task, could be explained by a biased effect of the fasting procedure applied prior to the training session to reduce the weight of the animals and induce motivation for food, this may have affected the performance of the task (Sohal & Forster, 2014). In addition, fasting can also increase responsivity to food reward (Stice et al., 2013) which can cause a possible biased effect. In conclusion, our results suggest that an olfactory food-reinforced task such as ODT is not the most suitable task for assessing the effects of a CR diet during aging due to the fasting that is needed before learning in order to motivate the animals to respond to the food reward. Further investigation is needed to confirm this hypothesis.

By the other hand, our results in the long-term MWM memory test demonstrated that CR might be a good intervention to improve spatial memory, as the performance of CR group was better than that of *Ad Libitum* animals and similar to the young group. The dietary intervention attenuated the age-related cognitive impairment detected in this spatial task in old animals in agreement with previous reports (Geng et al., 2007; Gyger et al., 1992; Portero-Tresserra et al., 2018; Stewart et al., 1989). Furthermore, the beneficial effects of the CR on MWM may not be

attributable to motor disabilities or anxiety disorders, since no differences in these control variables were found in any group or any phase. Regarding MWM learning, no effect of CR was found in the acquisition sessions, as previous literature predicted (Geng et al., 2007; Kuhla et al., 2013; Nakamura & Ohno, 1995; Stewart et al., 1989; Wang et al., 2007). Thus, both *Ad Libitum* and CR groups exhibited age-related cognitive decline across the learning phase (Adams et al., 2008; Portero-Tresserra et al., 2018), although all the groups of animals learned the task as their latency to find the platform decreased over time. In conclusion, 25-30% CR over lifetime seems to be beneficial for short and long-term memory tasks that do not require food as a reward or a prior fasting protocol such as ODT.

Memory improvement in old CR rats might be associated with beneficial effects of the diet on brain aging

In general, our results showed that CR enhanced long-term memory in the MWM, but not in the ODT. Regarding MWM long-term retention, the behavioral findings of our experiments support the hypotheses that CR might delay the age-related cognitive decline of spatial memory (Adams et al., 2008) and one of the underlying biochemical mechanisms that are involved in the performance of this task might be monoamines levels in the brain. In general, these NTs are linked to memory processes, which are partially regulated by their neuromodulatory activity (González-Burgos & Feria-Velasco, 2008; Kemp & Manahan-Vaughan, 2008). In our study, HPC 5-HIAA levels correlated with long-term spatial memory in the MWM, showing that animals with higher levels of this metabolite tended to recall the task better than animals with lower levels of 5-HIAA, in agreement with previous results (El-Falougy & Benuska, 2006; González-Burgos & Feria-Velasco, 2008). Moreover, FC DA and DOPAC correlated with MWM retention. In fact, both aged groups of animals presented lower levels of DOPAC and DA in the FC compared to young animals. These NTs and metabolites are down regulated during aging (Ponzio et al., 1982).

Another of the underlying brain mechanisms that might be involved in MWM memory consolidation is the levels of AMPAr subunits. It has been previously (Liang et al., 1994) demonstrated that those receptors in the HPC are related to memory processes. Our outcomes revealed that AMPA subunits 1 and 2 are diminished in *Ad Libitum* animals compared to both CR and young groups or rats. In addition, AMPA1 subunits in the HPC correlated with MWM recall in the test, demonstrating that animals with higher levels of this subunit performed and remembered the location of the platform better than animals with a lower level of AMPA1. Moreover, glucose and IGF-1 blood plasma levels are also linked to memory processes. Our results showed a negative correlation between glucose and long-term hippocampal memory in the MWM, as previously demonstrated (Malone et al., 2008), although no differences in glucose

levels were found between groups in our experiment. In addition, IGF-1 is essential for learning and memory processes and the age-related loss of this hormone may contribute to the cognitive impairment in adult rats (Lupien et al., 2003). Our results indicated that, as expected in young animals, IGF-1 levels were increased. Moreover, a relation between IGF-1 levels and long-term memory retention was observed in our data, which might explain the better performance of the Young group in the MWM task.

The MWM acquisition process was accomplished by all the groups, although young animals did it faster. This process is linked to NMDAr and AMPAr (Wang et al., 2013). In fact, in our study a correlation between AMPA1 in the HPC and the time to find the platform in the last acquisition session of the MWM was found, indicating that subjects with higher levels of those receptors learned the task faster. Interestingly, *Ad Libitum* animals showed lower levels of AMPA1 subunit in the HPC than the CR old and young group. Moreover, an association between NMDAR2A in the FC and acquisition in the MWM was detected, demonstrating the key role of this subunit in the acquisition of the task, as previous investigations have reported (Wang et al., 2013). Furthermore, these outcomes may also be linked to corticosterone levels. High levels of this hormone might impair the MWM learning (Luine et al., 1994) and, as our results confirmed, corticosterone levels are increased in aged rats (Sapolsky et al., 1985). In addition, a negative correlation between levels of corticosterone and performance in the last acquisition session in the MWM was found. This result indicated that animals with higher levels of corticosterone showed an impaired learning process, which may explain the worse performance of the aged animals.

Finally, ODT does not seem to be a suitable task for analyzing memory in a CR intervention study in old rats, although underlying biochemical mechanisms might explain the rationally of this impediment. Previous experiments (Tronel, 2002) have demonstrated that levels of NMDAr are involved in FC dependent tasks such as ODT. Our results confirmed that animals with higher levels of NMDAR2A and AMPA1 in the FC tended to perform better in the ODT acquisition than rats with lower levels of these subunits. However, the biochemical results in our experiment indicated that CR did not affect the levels of NMDAr subunits in HPC and in the FC and AMPAr in the FC, as no differences between groups were found. Moreover, our findings confirm the key role of those receptors in the ODT memory task, as previously demonstrated (Tronel, 2002). Interestingly, in our experiment, AMPA1 levels in the HPC of *Ad Libitum* rats were down regulated and CR was able to attenuate this age-related decline. Therefore, this data might explain the slightly worse performance of *Ad Libitum* old rats when compared to the other two groups in the retention session.

In conclusion, the results from our study confirmed that, in general, a lifelong CR diet is able to attenuate the age-related cognitive decline detected in old rats. Moreover, these beneficial effects of CR on memory might be related to the enhancement of the monoaminergic and glutamatergic neurotransmission in the aged brain. Moreover, plasma levels of hormones such as corticosterone and IGF-1, and other molecules such as triglycerides and glucose, are also involved in the improvement of the behavioral performance in the CR rat. Future research of the effects of CR on the aging brain will be necessary and of great interest to fully understand the effects of such dietary intervention on memory decline to search for new behavioral habits that assure a long and healthy life in humans.

VII. CONCLUSIONS

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1. Results showed that all animals were in good health with no malnutrition, as demonstrated by the comparable blood plasma levels of insulin and cholesterol and normal age-pattern levels of glucose and corticosterone in both aged groups.

2. The 25-30% CR intervention was able to modify the age-related increase in plasma leptin levels, since CR animals showed comparable levels of this hormone to the young control group.

3. The biochemical data confirmed the hypothesis of monoaminergic age-dependent decline that was ameliorated by CR. Aged animals presented reduced levels of serotonergic transmission in the HPC and the dopaminergic pathway in the striatum, which was reversed by CR intervention.

4. CR partially modifies the age-related loss of glutamatergic transmission as this dietary intervention enhanced the age-related loss of AMPAr in the HPC, although it failed to modify the levels of NMDAr in the FC and HPC and AMPAr in the FC.

5. Although CR was not able to modify SYP levels in the different brain regions analyzed, we cannot rule out this dietary intervention having a positive effect on synaptic plasticity during aging.

6. A lifelong hypocaloric diet improved short-term memory (Object recognition in Y maze) and long-term memory (Morris Water Maze) of old rats since CR animals' performance did not differ from that of the young rats. These results support the hypothesis that dietary interventions such as CR may prevent or slow down the progression of age-related cognitive deterioration.

7. The old *Ad Libitum* group of rats showed a worse performance in the object recognition in the Y maze and the MWM tasks when compared to the young and CR old groups of animals, showing that short-term and long-term memory decline are attributable to the aging process itself.

8. Olfactory food-reinforced tasks, such as ODT, are not the most appropriate task to evaluate the effects of a CR diet in rodents during aging due to the fasting needed to induce motivation for food.

9. The results obtained in the different tasks cannot be attributed to other variables such as anxiety and motor activity since no differences between Young, old *Ad Libitum* and old CR rats were found.

10. The correlation between plasma levels of hormones, lipids and proteins and the rodents' behavioral execution suggested that the impaired execution of *Ad Libitum* aged animals in a long-term memory task may be due to the long-term *Ad Libitum* diet, which worsened health parameters.

11. The correlations between the monoaminergic systems and the behavioral data demonstrated that the serotoninergic and dopaminergic pathways are related to the rodents' long-term spatial memory execution. These results suggested that CR might contribute, at least in part, to the beneficial effect in learning and memory processes.

12. The relation between glutamatergic receptors subunits and the behavioral data confirmed the implication of those receptors in long-term memory processes. These results suggested that CR might positively contribute, at least in part, to learning and memory processes which seem to be impaired in aging.

VIII. BIBLIOGRAPHY

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