Ndr2 deficiency prevents spatial memory decline and proteostasis alterations in the ageing hippocampus

Thesis

For the degree of

Doctor rerum naturalium (Dr. rer. Nat)

approved by the Faculty of Natural Sciences of Otto von Guericke University Magdeburg

by M. Sc. Miguel del Angel

born on August 12, 1989 in Mexico City

Examiner: Prof. Dr. Oliver Stork

Dr. Vassiliki Nikoletopoulou

Submitted on December 19, 2022

Defended on September 22, 2023

INDEX

TABLE OF FIGURES

ZUSAMMENFASSUNG

M. Sc. Miguel del Angel

Die Serin-Threonin-Kinase Ndr2 reguliert die dendritische Verzweigung und die Integrin-Signalgebung. Neben anderen zellulären Prozessen spielt sie auch eine wichtige Rolle bei der Autopagie Regulation im erwachsenen Gehirn. Es ist bekannt, dass eine Autophagie Dysregulation ein wichtiger Faktor bei der Entstehung altersbedingter neurodegenerativer Erkrankungen ist. Darüber hinaus ist eine ordnungsgemäße neuronale Autophagie von wesentlicher Bedeutung für die Gedächtnisbildung und verhindert eine Verschlechterung der kognitiven Funktionen bei älteren Menschen. Frühere Arbeiten unserer Gruppe haben gezeigt, dass Ndr2-KO-Mäuse Defizite im räumlichen Gedächtnis und bei verwandten Prozessen wie Langzeitpotenzierung und Spine-Bildung durch die Aktivierung von Integrinen aufweisen; dies zeigt, dass Ndr2 auch an Plastizität und Gedächtnis beteiligt ist. Dennoch bleibt die Frage offen, ob Ndr2 an der altersbedingten Autopagie Dysregulation und dem damit verbundenen kognitiven Abbau beteiligt ist.

In Kapitel 1 wurde der autophagische Fluss in Hippocampus-Neuronen von Ndr2 WT- und KO-Mäusen untersucht und der basale autophagische Fluss im dorsalen Hippocampus von erwachsenen Mäusen bewertet. In Kapitel 2 wurde ein Modell standardisiert, in dem Autophagie durch die Verabreichung des Disaccharids Trehalose induziert werden kann. Die Auswirkungen der Hochregulierung von Autophagie auf das vom Hippocampus abhängige Gedächtnis von jungen und alten Mäusen wurden untersucht. In Kapitel 3 wurde das hippocampusabhängige Gedächtnis von 26 Monate alten Ndr2 WT- und KO-Mäusen unter Kontrollbedingungen und unter Trehalose-Behandlung untersucht. In Kapitel 4 wurden die Proteinveränderungen, die während des Alterns im dorsalen Hippocampus von Ndr2 WT- und KO-Mäusen auftraten, untersucht.

In dieser Arbeit wurde gezeigt, dass Ndr2 die Autophagie in Hippocampusneuronen und im erwachsenen Hippocampus reguliert. Es wurde auch gezeigt, dass es die Lokalisierung von Autophagosomen innerhalb der Neuronen reguliert. Außerdem wurde festgestellt, dass die Verabreichung von Trehalose Autophagie nur im dorsalen Hippocampus alter Mäuse hochreguliert und das räumliche Gedächtnis und die Navigation beeinträchtigt. Es wurde beobachtet, dass alte Ndr2-KO-Mäuse Defekte der basalen Autophagie aufweisen, welche das räumliche Gedächtnis verbessern, und dass die Hochregulierung von Autophagie durch Trehalose das räumliche Gedächtnis beeinträchtigt. Schließlich konnte gezeigt werden, dass ein Ndr2-Mangel Proteomveränderungen im alternden dorsalen Hippocampus verhindert, insbesondere in Bezug auf Entzündungen, mitochondriale und ribosomale Funktionen sowie synaptische Übertragungswege.

ABSTRACT

M. Sc. Miguel del Angel

The serine-threonine kinase Ndr2 regulates dendritic arborization and integrin signalling. Among other cellular processes, it has also been strongly implicated in the regulation of autophagy in the adult brain. It is known that autophagy dysregulation is one major factor in the onset of age-related neurodegenerative diseases. Even more, proper neuronal autophagy is essential for memory formation and prevents deterioration of cognitive function in the elderly. Previous work from our group showed that Ndr2 KO mice display deficits in spatial memory and related processes, such as LTP and spine formation through the activation of integrins; these demonstrate that it is also involved in plasticity and memory. Nonetheless, the question of whether Ndr2 is involved in age-related autophagy dysregulation and the cognitive decline associated with it still remains.

In Chapter 1, the autophagic flux was evaluated in hippocampal neurons from Ndr2 WT and KO mice and the basal autophagic flux in the dorsal hippocampus of adult mice was assessed. In Chapter 2, a model in which autophagy can be induced by the administration of the disaccharide trehalose was standardized. The effects of autophagy upregulation in the hippocampal-dependent memory of young and old mice were evaluated. In Chapter 3, the hippocampal-dependent memory of 26-month-old Ndr2 WT and KO mice in control conditions and under trehalose treatment was assessed. In Chapter 4, the protein changes that occurred during ageing in the dorsal hippocampus of Ndr2 WT and KO mice were evaluated.

In this thesis, it was demonstrated that Ndr2 regulates autophagy in hippocampal neurons and the adult hippocampus. It was also demonstrated that it regulates the localization of autophagosomes within the neurons. Also, trehalose administration upregulated autophagy only in the dorsal hippocampus of old mice and impaired spatial memory and navigation. It was observed that old Ndr2 KO mice have defects in basal autophagy but improved spatial memory and that upregulation of autophagy with trehalose impaired spatial memory. Finally, it was demonstrated that Ndr2 deficiency prevents proteome alterations in the ageing dorsal hippocampus, particularly in inflammation, mitochondrial, ribosomal function and synaptic transmission pathways.

INTRODUCTION

Biology of Ageing

Ageing is one fundamental aspect of the human condition, as it is the final step of our development. Like almost any other living being, we grow up watching others become old and age, We see what ageing does to their body and minds, and finally, we experience it first-hand. This simple knowledge is so deeply rooted in our unconscious understanding of life that we do not need to go to a checklist or make any conscious effort to identify an old living being, from a toddler or an adult; it implies the same unaware effort as recognizing something alive, from something that never was. Nonetheless, ageing is arguably one of the less understood aspects of not only human biology but biology in general, on par with the origin of life itself. So many open questions have been proven very difficult to address, such as: What really is ageing? Is ageing the same among different organisms? When do we start to age? Is ageing the cause of age-related diseases or a consequence? Can ageing be prevented? And lastly, why do we age?

The number of studies in the field of ageing has increased substantially in the past three decades, as it reflects an increased concern of society and policymakers for the middle-term future of human health and how to deal with an increase in the elderly population. It is expected that the percentage of the population worldwide aged 65 and over will increase from 12% in 2015 to 22% in 2050 and that it will reach more than 30% in some developed countries[1]. Paradoxically, the expectation is that this increase in life expectancy will not be mirrored by an increase in healthspan, which is why there´s a growing global concern for the development and discovery of drugs and interventions that delay the age of onset of frailty that reduce the severity of age-related disease, thus increasing healthspan.

Even though the main objective of the current mainstream gerontobiology (the biological study of ageing) is to develop therapies to treat age-related diseases, increase healthspan or even lifespan, understanding the fundamental core aspects that underlie ageing is highly relevant, and it has been proven as challenging as finding a way to stop ageing. This is not trivial or a coincidence because, from a certain point of view, it may be impossible to manipulate and intervene in a process so complex as ageing when we do not even understand what it really is. Particularly in the field of gerontobiology, the difference between those who ask the questions of how? (molecular biologist) and those more focused on the why? (evolutionary biologists) seems to be larger than in any other field of biology. The reason

for that is that a superficial understanding of ageing could seem like an evolutionary paradox because ageing is fundamentally detrimental to the survival of organisms, hence natural selection should remove it over time, and a more mechanistic explanation of ageing is easier to interrogate, easier to understand and answers more questions than what it rises.

From a biological point of view, ageing has been broadly defined as the loss of biological performance in function of time that inevitably increases intrinsic mortality. Still, the reality is that not all organisms age at the same rate or go through this stage similarly. The current paradigm that tries to understand ageing encompasses mainly two different but not necessarily incompatible points of view. The first one considers ageing as an accumulation of damage in the cells, which is a direct consequence of time. The second, and currently the most accepted one, comes from the coffers of evolutionary biology, and it is often simply and unpretentiously called the evolutionary theory of ageing.

In short words, the evolutionary theory of ageing proposes that antagonistic pleiotropic genes, which are those that have multiple and opposing traits during the lifespan of organisms, are strongly favoured by natural selection if they increase fitness in early life stages, regardless of whether they are detrimental after reproduction has ceased. These traits can vary from species to species, and even though many commonalities exist between ageing organisms, there are also many characteristics unique to different biological entities. The ageing phenotype is as broad as the many different organisms that exist on this planet, and that is no coincidence at all because the traits that increase the fitness of a certain species or population are unique to them, and therefore the pleiotropic effects that appear in ageing are as well. A high tolerance to an anoxic environment that causes oxidative damage in the liver during ageing is useful when you are a mole living in a burrow, but not quite much if you are a whale in the open sea! The ease in which ageing is shaped across species is particularly evident in mammals, and as a result, there is a wide spectrum of ageing phenotypes, such as shrews that age fast and have life expectancies of no more than one year[2]; species that live decades without any evident symptoms of ageing or an increase in mortality[3] such as the naked mole rat[4]; and species that can live more than 100 years but that show a wide array of age-related diseases, increased frailty, and inability to reproduce for almost half their late lifespans as we humans do[5]. Therefore, the ageing process is under constant evolution and is shaped by a complex interaction of biotic and abiotic factors, such as the weather, food and water availability, predation or competition, that exert a wide array of selection pressures throughout all the life history of living beings. One of the most well-studied examples of the effect of the environment and predation, often considered the two most factors that shape the evolution of age

phenotypes, could be observed in the widely studied island opossum *Didelphis virginiana,* which as a consequence of the absence of predators in their habitat, evolved a retarded ageing phenotype and increased lifespan, this is in harsh contrast with the fast-ageing mainland population[6] that shares its habitat with many predators and other small mammals that compete for food and living space. The incredible thing is that this extreme difference in maximum lifespan, around six years for the island population, and 2 for the mainland one, evolved after only 5 thousand years of isolation! Indeed, there is great insight in studying ageing from an evolutionary point of view because if evolution can shape in such a fast (in evolutionary times) and in a wide manner (across several orders of mammals) the ageing process, then it is understandable to suppose that there are some more simple and general cellular mechanisms underlying the ageing phenomena.

Hallmarks of ageing

Truly, a great effort has been put into understanding the basic biological principles of ageing across all fields of biology, chemistry and physics. One of the breakthroughs made to better understand how do we age? are the so-called hallmarks of ageing. They encompass a catalogue of known cellular processes that are thought to drive forward ageing because it has been shown in several organisms and biological models that the appearance of these altered cellular processes often correlates with the decline of physiological performance, ageing, and age-related diseases. But the fundamental aspect of the hallmarks of ageing is that the pharmacological or genetic modulation of them delays and ameliorates health, age-related diseases and even in some cases, they increase lifespan[7]. These events can be categorized as wide functional cellular changes such as senescence or stem cell exhaustion, alterations directly related to changes in the translational machinery like genomic instability and epigenetic alterations and lastly, changes in core processes involved in cellular metabolism like altered nutrient sensing and loss of proteostasis.

It is also a fact and currently accepted as the paradigm that even within the same organism, the different organs of the body age at different rates and are susceptible to different kinds of stress and normal physiological processes during our daily lives. The result is that within one individual, the different organs go through different kinds of stress and damage, and the therapies aimed to ameliorate ageing should be addressed accordingly. The brain is arguably one of the most susceptible organs to damage and stress. Among the main age-related changes that the brain experiences are the alterations in Ca^{2+} homeostasis, accumulation of protein aggregates, increase in reactive oxygen species (ROS) and

increased neuroinflammation. In the brain, the most obvious evidence of age-related damage and deterioration is, of course, changes in behaviour and loss of cognitive capabilities. The deterioration of cognition during ageing has been widely studied, and it can arguably be said that it's the main focus of current mainstream neurobiology and the search for means to prevent memory loss, dementia, and changes in cognition, one of its main goals. In that regard, many novel therapies that directly target one or several of the hallmarks of ageing are under current investigation. For example, the possibility that neural stem cells could be directly transplanted in mice and improve memory has been recently demonstrated to be true and, very likely, a therapy for humans, given that the stem cells can be transplanted intranasally[8].

Given that the brain is a very oxygen-demanding organ, it also produces a lot of ROS. Even though ROS have been linked to signalling and development, it has also been shown widely that young individuals have less ROS and higher production of antioxidant molecules in the brain that increase the oxidation of biomolecules, oxidative stress and, ultimately, impair cognitive function[9]. For that reason, one of the most common approaches that has already been proven to improve memory, and prevent memory loss and even the pathological neurodegeneration in mouse models of Alzheimer's disease (AD), is to reduce the levels of ROS. Interestingly the ROS production positively feeds the dysregulation of other hallmarks of brain ageing, like altered DNA methylation, which results in a change in the brain transcriptomic landscape, alterations in the mitochondrial function, which also relates to altered energy metabolism, and so on[10]. The alteration of proteostasis in the brain is also one of the most studied hallmarks of ageing and one that has been linked the most to the loss of cognition, particularly in age-related neurodegeneration. For instance, it is known that the accumulation of protein aggregates is one of the main cellular correlates and a commonality of several age-related diseases like AD, Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), Huntington´s disease (HD), or frontotemporal dementia. It has even been shown that an increase in protein aggregates in healthy individuals without neurodegenerative diseases negatively correlates with memory function[11]. Another strong argument is that the upregulation of autophagy, one of the main proteostatic pathways of the cell, has been proven to prevent memory loss in ageing mice[12].

Proteostasis refers to the chronical decrease of the cell´s ability to ensure the correct folding, stability, transport, and eventual degradation of proteins. The principal mechanisms by which the cell accomplishes the latter are thanks to the cell´s main degradative pathways: The ubiquitin-proteasome system (UPS) and lysosomal degradation through autophagy. In contrast to the UPS, which is only

capable of degrading proteins, autophagy is the only known mechanism that the cell has to degrade proteins and organelles, such as the endoplasmic reticulum, mitochondria, peroxisomes, ribosomes or even the nucleus[13], because of that, it should be no surprise that almost in all eukaryotes, and particularly in animals, the alterations in autophagy are deeply associated with ageing and are thought to be one of the main contributors to lifespan and healthspan[14].

Autophagy

Three different mechanisms for autophagic degradation have been characterized so far: Microautophagy, chaperone-mediated autophagy (CMA), and Macroautophagy. Microautophagy is probably the less studied form of autophagy in vertebrates, as it was previously thought to occur only in yeasts and plants[15]. During this process, the lysosome or the vacuole, in the case of plants, directly engulfs a small portion of the cytoplasm, hence the term Microautophagy. This process has not been widely studied in mammals, but what is known is that it makes use of some of the core autophagy machinery and the ESCRT protein complex to sort cargo into the invaginating lysosome[16], unfortunately, as a consequence of a deeper lack of understanding of Microautophagy, the relevance of this process in ageing is still unknown, in harsh contrast to both CMA and Macroautophagy, that have been proven to be of particular research interest in the biology of ageing. CMA is a highly selective process in which the proteins to be degraded are delivered to the lysosome directly by the chaperone HSC70, which recognizes and binds directly to a highly conserved aminoacidic motif in the target proteins, called the KFERQ motif because of its sequence, acting as a scaffold between the cargo and the lysosomal receptor LAMP2A. Once docked, the protein unfolds, LAMP2A homotrimerizes, and translocates the protein into the lysosomal lumen, where it will eventually be degraded[17].

On the other hand, macroautophagy is the most well-studied form of autophagy, up to the point that is often termed in the literature as homonymous with the whole autophagic pathway. It refers to the formation of a double membrane vesicle named autophagosome around the cargo to be degraded, the fusion of the autophagosome with the lysosome and the eventual degradation of the contents through the action of lysosomal hydrolases. It´s worth mentioning that even though autophagy is mostly regarded as one of the main catabolic pathways of the cell, second only to the UPS, it has many non-canonical functions that are not always directly related to the degradative form of autophagy, such as intracellular transport and secretion. But even if we don't consider the non-canonical forms, and we highlight only the degradative autophagy, it is clear that it plays a central role in cellular signalling

because it bridges degradation and recycling (catabolism and anabolism), so it's often considered a crossroads of many pathways of energy metabolism[18], this is because starvation is one of the main inductors of autophagy[19], that causes a bulk degradation of cytoplasmic contents that ensures cell survival. After all, the recycling of macromolecules helps, in turn, to restore the nutrient levels to their basal state preventing an energy and nutrient imbalance, and eventually cellular death. This has been widely proven to be true for the replenishment of carbohydrates, lipids and aminoacids[20], being the latter particularly sensitive to the reduction of nitrogen levels[21]. It is important to highlight that this effect is cell-dependent because the energy requirements of the different cell types are unique to each of them. For instance, in the liver, autophagy is highly induced by glucose and aminoacid deprivation, but not quite so much upon lipid deficiency[22], or skeletal muscle cells, where autophagy can be easily induced by nutrient deprivation (glucose, aminoacids, lipids), but also by exercise, in contrast with other organs. The brain, and particularly the neurons, are highly sensitive to stress and nutrient deprivation, so other mechanisms have evolved to keep the nutrient levels stable, that prevent a starvation-like state and the induction of autophagy by these means, whereas signalling molecules such as Ca^{2+} or neurotransmitters have been shown to induce autophagy for neuronal-unique functions[23]. Although the role of autophagy in maintaining the homeostasis of nucleic acids upon nutrient starvation has not been thoroughly explored, it is known that the recycling of DNA and RNAs can also occur within the autophagosome, the former of particular importance under the activation of the DNA damage response (DDR)[24], and the latter under basal autophagy[25]. Autophagy induction upon nutrient deprivation is, thus, an essential pro-survival mechanism, as it has been shown that impairment in the autophagic machinery during starvation proves to be lethal in a wide range of organisms, such as protists, nematodes and even mice[26–28].

On the other hand, autophagy occurs not only as a non-specific bulk process but can also be highly selective because, along with the UPS, it is one of the main pathways by which the cell can get rid of unfolded, damaged or un-needed proteins, this kind of autophagy, contrary to bulk non-selective, it is particularly important for post-mitotic cells like neurons that can´t get rid of the excess aggregates or damaged organelles by cell division. Selective autophagy is achieved by a conjugation system that uses scaffolding proteins that anchor the target proteins to be degraded on the autophagosome membrane. It is also very noteworthy that autophagy is the principal (if not the only) mechanism that eukaryote cells have to degrade both insoluble protein aggregates and complete organelles.

Molecular mechanisms of autophagy.

Because autophagy is so important to maintain homeostasis, it is no surprise then, that it exists under tight regulation. The initiation, nucleation of the autophagosome, elongation, fusion with the lysosome, degradation and the eventual reconstitution of the lysosome are regulated by several protein complexes highly conserved between the eukaryotes. The first autophagy-related proteins (ATGs) were described in yeast, and since then, homologous proteins have been described for numerous ATGs in plants and animals.

Autophagy initiation

Although there are peculiarities in the autophagy mechanisms between different organisms, and even between cell types, generalities do exist: In mammals, the protein complex consisting of ATG13, FIP200, ATG101 and the serine/threonine kinase ULK1 or its homologue ULK2 (Atg1 in yeasts) serves as the first upstream complex of the autophagic pathway[29]. This protein complex, mainly through the phosphorylation of ULK1/2, integrates the signals received by the AMP protein kinase (AMPK) and mTOR. Under basal conditions, ULK1/2 auto-phosphorylates at S1042 and Y1046, which promotes its degradation in a negative feedback loop. This regulation prevents excess degradation in normal physiological conditions and also serves as a break during prolonged periods of starvation[30]. In contrast, under glucose insufficiency, AMPK becomes active by an increase of cyclic AMP (cAMP) levels, and then it phosphorylates ULK1 in the S317 and S777[31]. This phosphorylation stabilizes the protein complex, favouring the translocation of the whole complex into proximity to the pre-autophagosomal structure (PAS) in yeasts or to the autophagosome formation site in other eukaryotes. In mammals, it has become more accepted that there is not one specific compartment or structure within the cell that acts as the sole autophagosome formation site, even though the endoplasmic reticulum (ER) is often regarded as the main site for autophagosome formation, specifically the endoplasmic reticulum exit sites (ERES) that exist in structures called omegasomes, every day there is more evidence that the Golgi[32], the mitochondria[33], the late endosome, the plasma membrane[34] and even the nucleus[35,36] serve as membrane donors and autophagosome formation sites. In harsh contrast to the activation of ULK 1/2 by cAMP by the drop of glucose levels, when mTOR is active, it phosphorylates ULK1/2 in the S757, which prevents the interaction with AMPK[31], thus preventing the stabilization of the complex and also promoting the autophosphorylation of ULK1/2 for degradation.

Nucleation and elongation

The Beclin1 complex is one of the principal targets of ULK1/2. It comprises Beclin1, the vacuolar sorting protein 34 (VPS34), a class III phosphatidylinositol 3 Kinase (CIII-PI3K) and AMBRA1. The latter serves as a scaffold of the complex with the microtubules. Upon its phosphorylation by ULK1/2, it releases Beclin1 and VPS34. It allows them to interact with ATG14, which promotes the translocation of the complex, where it will interact with the membrane-bound ATG15, which will anchor it to the nucleating membrane[37], also called the isolation membrane. Besides the phosphorylation of AMBRA1, ULK1/2 also phosphorylates Beclin1 in the Ser15 and Ser30, which is essential to maintain the stability of the complex and for the production of phosphatidylinositol-3 phosphate by VPS34[38,39] once the complex reaches the isolation membrane. This phosphoinositide is required to anchor yet another protein complex consisting of WIPI2 (ATG18 in yeast), ATG16L1, ATG12 and ATG5. This complex is of great importance because it is required for the lipidation of the adaptor-conjugating proteins required for the recognition of autophagic cargo such as Atg8 in yeast or its orthologues: the members of the microtubule-associated protein1 light chain 3α (LC3) or GABA-A type associated protein (GABARAP) family of proteins. Although LC3B is apparently the predominant and definitely the most studied form of the adaptor-conjugating proteins, in mammals, there are at least three other members of the LC3 family of proteins (LC3Aa and LC3Ab, and LC3C), and three members of the GABARAP family of proteins (GABARAP, GABARAPL1 and, GABARAPL2). It is known that all of them have a high sequence homology and redundant functions in recognizing cargo-adapting proteins. Still, they also have unique cell-dependent functions as they are under the control of different transcription factors and are regulated differently through post-translational modifications[40].

Nonetheless, all proteins must go through the same processing steps before they can be covalently lipidated with phosphatidylethanolamine (PE) and conjugated into the isolation membrane, where they will exert their function in the cargo recognition mechanism. The first step in the conjugation system is a proteolytic cleavage of LC3 mediated by the only protease involved in the regulation of autophagy, the cysteine protease ATG4. This cleavage exposes a glycine residue in the C-terminus end of the protein. Once cleaved, LC3 (now called LC3-I or GAPARAP-I whether it be the case) is adenylated by ATG7, an E1 Enzyme-like that facilitates its transfer from an ATG7/LC3 intermediate complex to the E2-Enzyme like ATG3[41] . This protein is quite remarkable because it has a curvature sensing domain, meaning that this transfer will only occur in highly curved membranes such as the isolation membrane[42]. Once in close proximity to the isolation membrane, the ATG5/ATG12 complex mediates the transfer of LC3-I from ATG3 to the amino group of the PE, this lipidated form of LC3 is often referred either as LC3-

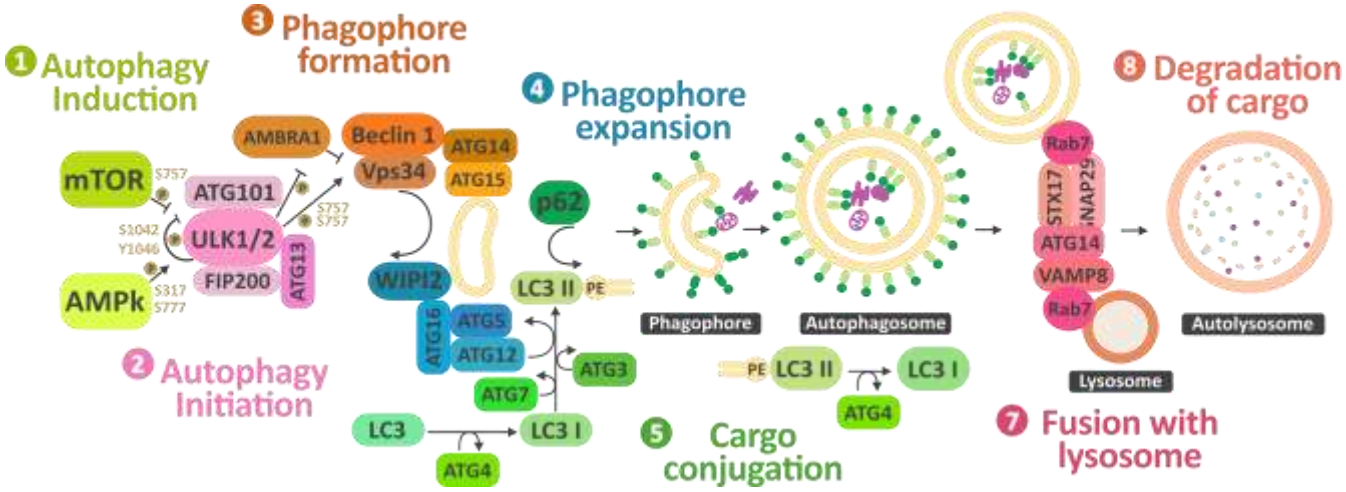
PE or LC3-II (conversely, GABARAP-PE or GAPARAP-II). Once in the isolation membrane, cargo adapter proteins such as p62/SQSTM1, NBR1, optineurin/OPTN, Huntingtin/HTT, ALFY, NDP52 and Atg 39 or Atg40 in yeast will act as a scaffold between LC3-II and the cargo to be degraded. This is achieved through a C-terminus motif called LC3 interacting region (LIR) and an N-terminus ubiquitin-association (UBA) domain that recognizes ubiquitinated amino acids in the target cargo. Although the LIR sequence can be highly variable between proteins and even organisms, the consensus sequence is usually considered to consist of [W/F/Y]xx[L/I/V][43]. Like the adaptor-conjugating proteins, the cargo adaptors are considered to have both redundant functions but also to participate in different types of autophagy, such as aggrephagy (p62, ALFY)[44] mitophagy (OPTN, NDP52), reticulophagy (p62, NDP52, Atg39)[45,46], nucleophagy (p62, Atg39)[46,47] and xenophagy (p62, OPTN, NBR1)[48]. Once the cargo is tethered to the isolation membrane (now termed phagophore) through the adapter protein, the membrane will expand around the cargo. It is generally accepted that, at this point, the phagophore has no clear polarity and that it will acquire it as it expands. Another important regulatory mechanism during this step is the cleavage of the unconjugated LC3-II from the phagophore´s membrane by ATG4. This cleavage reconstitutes the pool of LC3-I[41] and helps give the phagophore a clear polarity because it prevents new cargo from attaching to what will be the outer membrane of the autophagosome. It is only once the phagophore fully encloses the cargo by this process that it is considered a mature autophagosome.

Fusion with the lysosome

One of the last steps is the dephosphorylation of phosphatidylinositol-3 phosphate by species-specific phosphatases, this dephosphorylation primes the outer membrane of the autophagosome to eventually fuse with the lysosomal membrane[49]. Rab7 is recruited to the outer membrane in a less well-understood process, which serves as scaffolding between the autophagosome and cellular motor proteins. In a very similar fashion, as it is known to occur in the lysosomes, the movement towards the nucleus occurs mainly through the microtubules and is dependent on the association of RAB7 with dynein. An interesting regulatory mechanism is that this association is inhibited in low nutrient conditions, in which interaction with kinesin is favoured, along with transport to the periphery. Rab7 is also an important player in autophagosome-lysosome fusion, as it serves as a nucleation point for SNARE proteins. There is a great diversity of SNAREs, and though its involvement in autophagosome-lysosome fusion is context and species-dependent, the most important ones are STX17, STX12 and SNAP29 in the autophagosome and VAMP8 in the lysosome. These SNAREs form complexes in the autophagosome and lysosome outer membranes that interact with each other through ATG14L, which brings the two vesicles together[50]. Once in close contact, the outer membrane of the autophagosome fuses with the lysosome, and the

lysosomal proteases degrade the inner membrane of the autophagosome and the cargo, including the adapter proteins and the membrane-bound LC3 II.

Neuronal Autophagy



Autophagy pathway at a glance. Autophagy induction depends mainly on two nutrient sensing pathways, AMPK and mTOR, which phosphorylate ULK1/2 to stabilize the complex and prevent or favour its degradation, respectively. The stabilized ULK1/2 protein complex initiates autophagy by phosphorylation of AMBRA 1 and Beclin 1, which produces the translocation of the protein complex formed by Beclin1 and Vps34 to the isolation membrane. Once in there, Vps34 incorporates phosphatidylinositol-3 phosphate, which allows a protein complex formed of ATG5 and ATG12 to incorporate the previously activated LC3 to phosphatidylethanolamine to the phagophore, LC3 II in turn, interacts with cargo adapter proteins like p62, that tether the autophagic cargo to the expanding phagophore only once closed it is considered a mature autophagosome. It is then when, SNARE proteins like Syntaxin19 and SNAP29 are recruited to the outer membrane of the autophagosome in a Rab7 dependent manner, and through ATG14, they interact with SNAREs on the lysosome that bring the two vesicles together and favour the fusion with the lysosome, where contents will be degraded by lysosomal hydrolases.

Neurons are post-mitotic cells, and as such, the degradation of damaged proteins and organelles poses a higher challenge because neurons are very complex in morphology and can acquire lengths of meters in some organisms, including humans. It is for those reasons that specific autophagy mechanisms have evolved in these cells to ensure a higher proteostasis quality control. One of the first peculiarities that were observed in neurons is that autophagy is spatially compartmentalized along the neuron. Autophagosomes nucleate in the distal axon, and they can travel both towards or against the soma of the neuron. This movement occurs thanks to cellular motors like dynein and kinesin, but at some point,

during early autophagosome maturation kinesin dependent movement becomes downregulated[51], favouring the retrograde movement towards the soma along the axon, where they get acidified and fuse with the lysosomes. It is accepted that the degradation of cargo occurs primarily in the soma and that disruption in the compartmentalization of autophagy is heavily associated with pathological conditions or stressors such as local depolarization[52]. Even though the mechanisms of autophagosome formation are in principle the same as in other cell types, in neurons, novel regulators that do not participate in autophagy in other cells have been observed, such as Endophilin A[53], that upon phosphorylation of LRRK2 promotes the formation of highly curved membranes[54] that are needed for ATG7 to transfer ATG3 and for the subsequent lipidation of LC3 II. Another interesting mechanism involves the regulation of ATG5 by Bassoon, a protein primarily expressed in the active zone. It was demonstrated that the expression of Bassoon inhibits ATG5 and, thus, the lipidation of LC3. Autophagosome formation in the distal axon helps maintain the balance between newly synthesized proteins and organelles in the soma, and the degradation of damaged proteins and old organelles in the distal axon where the quality control is of particular importance for the synaptic transmission, as it has been shown that activation of autophagy increases synaptic vesicle numbers, and the release of neurotransmiters[55]. Nevertheless, it is known that the retrograde movement also serves as a means of transportation for signalling molecules such as TrkB-bound BDNF within the neuron[56]. In contrast, within the dendrites, the microtubules have no clear polarity as it happens in the axon; for that reason, both anterograde and retrograde transport exist parallelly. This has also been associated with the different physiological functions that occur in dendrites during synaptic transmission and plasticity, although the peculiarities are still not completely understood.

Another peculiarity of the neurons is that autophagosomes under normal healthy conditions are seldom observed. This is mainly due to the fact that neurons have very efficient basal autophagy, so the autophagic vesicles do not tend to accumulate. This also means that neurons are cells in which autophagy is highly active, related to the neuronal-unique functions that autophagy acquires in synaptic transmission and neuronal homeostasis. It has been demonstrated that autophagic flux in the neurons exists in a very tight balance, much more than in any other cell type and that either too much autophagosome formation or not enough autophagic degradation can lead to neurodegeneration[57]. It has been shown that loss of this balance can lead to many different effects ranging from alterations in the dendrites, like reduced neurite branching, to neurodevelopmental diseases[58]. There´s also evidence that autophagy is required for synaptic pruning, maturation of motoneurons, and axonal growth, among others[59]. Evidently, the molecular pathways involved in the regulation of autophagy in the neurons are quite unique to them. We know that some molecules, such as BDNF, suppress the autophagic flux

through its receptor TrkB and the PI3K/AKT pathway[60], linking autophagy to memory to plasticity and memory formation. Also interestingly, it was shown that this process occurs under nutrient starvation, showing that nutrient sensing has different roles in the brain other than maintaining energy homeostasis. [61]In that regard, it has also been shown that starvation can also induce autophagy in other brain regions, like the hypothalamus, through the modulation of neuropeptides like agouti-related peptide AgRP, one of the most important peptides involved in the appetite response, and that if autophagy is inhibited, also the appetite response AgRP. So, from a certain point of view, even though autophagy doesn´t necessarily regulate energy metabolism within the neurons upon starvation, it does so in a systemic way. Another interesting modulator of autophagy in the brain, closely related to AgRP, is the neuropeptide Y (NPY), the most abundant neuropeptide in the brain. It has been widely demonstrated that NPY stimulates autophagy through the NPY1 and five receptors, and the PI3K AKT mTOR pathway, MEK/ERK and PKA signalling in cortical neurons[62] the hypothalamus[63], and also in the hippocampus, as has been demonstrated by previous unpublished data from our group, linking autophagy with hippocampal-dependent memory like spatial, working, episodic and fear memory. Another studied mechanism of autophagy activation in the neurons relates to the neurotransmitter NMDA. It is known that the recycling of the NMDA receptors through autophagy during long-term depression (LTD)[64–66].

Moreover, the accumulation of immature autophagosomes in the neurons has been observed in several AD mouse models, to the point that it has been proposed as one of the hallmarks of the disease[67]. Furthermore, in the brains of AD patients, there are reports of an abnormal accumulation of acidic vacuoles, most likely immature autophagosomes [68]. It is also known that Tau protein is a substrate of both autophagy and CMA; nevertheless, the pathological mutations that are associated with the aggregation of tau or even post-translational modifications associated with AD disease and other tauopathies hinder its degradation[69]. Tau is not the only protein aggregate in AD disease; in an *in vitro* AD model, it was shown that Beclin1 knockdown increases the intracellular aggregation of Amyloid-β peptide (Aβ) as well as its deposition, which was rescued upon autophagy upregulation through Beclin1 overexpression.

With regards to other neurodegenerative diseases, the accumulation of α- synuclein is regarded as a cardinal in PD, and although α- synuclein is mainly degraded through the proteasome system, it is also a well-known substrate of autophagy and CMA[70]. Overexpression of α- synuclein is enough to impair autophagy, as well as mutations that prevent its correct degradation. Accordingly, gene duplications that increase its expression are enough to cause PD[71]. Mitochondrial dysfunction is also another

important biological feature of PD. In regards to this, it is known that mutations on PINK or Parkin are linked to the familial form, and interestingly both proteins are master regulators of mitophagy. They bind to the ubiquitinated outer mitochondrial membrane and act as cargo adaptors; that´s why pathological mutations reduce the turnover of damaged mithochondria[72]. HD, in spite of being an autosomal dominant hereditary disorder, involves an autophagy impairment as well. The disease is caused by a varying-length polyglutamine expansion in the Huntingtin protein that accumulates and produces neurodegeneration. Although the mutated form is widely studied, its normal functional role is not completely understood. It is known that Knocking out the protein is lethal in mammals, as it prevents gastrulation, pointing out its biological relevance. There is also evidence that shows that huntingtin can act as an autophagic cargo adapter[73], and that it is necessary for the maturation of the autophagosome, with a particularly important role in the neurons[74]. With regards to ALS, it is known to be caused by impairment in autophagy degradation; mutations in the autophagic adaptors p62 and Optineurin are known causes of ALS[75], plus the accumulation of damaged organelles and an increase in Beclin 1 and LC3 levels have been observed in spinal cord samples of ALS patients[76], but moreover, it was shown that upregulating autophagy in an ALS mouse model overexpressing the superoxide dismutase 1 (SOD1) proved to be beneficial on the early stages of the disease, but not on later stages, which suggests that there is a complex interaction that involves autophagy in the pathological progression[77]. Although Neurodegenerative diseases are the most well-studied conditions, autophagy downregulation is also involved in other age-related diseases such as osteoarthritis[78], eye diseases like glaucoma, cataracts, macular degeneration[79] cardiovascular disease[80] and, very importantly, in diabetes[81].

Autophagy in cognitive ageing

It is clear, then, that autophagy plays an important role in neuronal biology and that alterations in the pathway lead to harsh effects like neurodegeneration and neurodegenerative diseases. Also, during ageing, there is a global loss of regulation of autophagy. For this reason, a lot has been speculated about the role of autophagy in memory formation, particularly during ageing, where there is a very evident loss of cognitive capabilities. There are many kinds of biological or psychobiological models that try to explain what memory is and how memories are formed, but one common point of agreement is that there are some memories that are short-lived and some others that remain for days, months or even years. Further on, both short and long-term memory can be categorized according to the types of memories that are related to it. This categorization is not trivial because it not only reflects correlations between memories of likewise nature but also a deep biological functional relation of neuronal events

that occur in constrained neurons in defined brain areas that associate with the formation and recall of such memories. This observation is important because, as was discussed before, not all the cells and organs (or, in this case, brain areas) are subjected to the same kind of stress and are not equally susceptible to the accumulation of damage over time; therefore the different kinds of memory are also affected differently during ageing[82]. In humans, we know that the most affected type of memories are those that depend on the hippocampus and the prefrontal cortex, like episodic memory; which refers to the memories of events and experiences, the declarative memory; the faculty of recalling punctual facts and information, and the spatial memory, which is the ability to remember the location of objects in space and to navigate to them [83–85]. It is widely accepted that spatial memory representation is carried out in the hippocampus by the place cells. And even though some models propose that the full neuronal mechanisms that underlie spatial memory formation involve the recruitment of other regions like the medial temporal, parietal, prefrontal lobes, entorhinal and prefrontal cortex[86], the correct functioning of the hippocampus remains of paradigm importance, as it has been thoroughly demonstrated that even small injuries in the dorsal hippocampus impair the formation of cognitive maps. In the context of neurodegenerative diseases such as AD, this observation remains consistent, as it is known that one of the earliest signs of degeneration is the loss of synapses in the dentate gyrus (DG) and impairment in spatial memory, but if the loss of synapses is prevented, the spatial memory is preserved as well[87].

Interestingly, even though autophagy plays such an important role in neurons, not much has been done in the field of cognition in ageing, but one of the most remarkable experiments in that field demonstrated that the upregulation of autophagy by activation of Beclin 1 directly in the hippocampus of old mice with a Tat-Beclin 1 peptide, prevented the loss of memory in a non-pathological context, and also that proper neuronal autophagy prevented the memory decline associated with ageing[12]. This held true for fear conditioning and spatial memory. Another experiment that pointed out a possible role of autophagy in spatial memory, involved subjecting caloric-restricted mice to learning in the Morris water maze, a test designed to test spatial memory and navigation. It was demonstrated that juvenile caloric-restricted mice found the escape platform of the maze faster than those with a normal diet on a high-fat diet[88], and that life-long caloric restriction increased the improvement in spatial memory even more[89]. This is a very interesting observation because first, it implies that modulation of autophagy at different stages of life history and or duration have different effects on memory and, second, that autophagy modulation might affect cognitive reserve.

All and all, autophagy seems a very promising target in the search for therapies aimed directly at the prevention of memory loss in the elderly, and finding new regulators and pathways that can be

intervened to increase autophagy during ageing in a way that can be easily translated to humans, is one of the major topics of interest for the ageing research.

Intervention of autophagy as an anti-ageing therapy

It´s clear that autophagy plays a central role in age-related diseases and that its intervention might prove useful in designing new treatments and drugs aimed at preventing the emergence of age-related diseases; nonetheless, it also plays a very important role in normal healthy ageing. It was demonstrated that during ageing, there´s a decrease in *Beclin 1* serum levels, but that in healthy centenarians (people aged over 100 years), *Beclin 1* levels mirror those found in young subjects[90]. In another similar study, it was shown that the autophagy-lysosomal pathway was one of the few upregulated pathways in centenarians, in contrast with middle-aged (45 to 78-year-old) adults, and not only that, but that high serum levels of *Beclin 1, Wipi1, ATG4, ATG18* also exist in younger family members[91]. One of the few ongoing longitudinal studies of ageing in primates demonstrated that caloric restriction, one of the main inducers of autophagy, improves healthspan and increases the survival of rhesus monkeys[92]. Another example comes from rapamycin, one of the most widely used activators of autophagy by the inhibition of mTOR. Rapamycin is often regarded as one of the first anti-ageing drugs. It has been demonstrated in several models, ranging from flies to rodents, that it prevents age-related cellular changes in neurons[93], age-related organ deterioration[94], and even increases lifespan in mice[95].

Macroautophagy is not the only one involved in age-related diseases. It has already been widely reported that in patients with HD, PD, AD, ALS or Frontotemporal lobar degeneration, there´s also downregulation of CMA, adding to the fact that several proteins involved in the onset or progress of the disease are well-known substrates of CMA, such as α-synuclein, Huntingtin, Tau and the leucine-rich repeat kinase 2 (LRRK2)[96]. It is known that the common pathological forms of these proteins fail to be fully degraded and instead start to form aggregates in the cytosol and lysosomal membrane, thus, impeding the degradation of other non-related CMA substrates. Another piece of evidence that supports the idea that CMA impairment is a hallmark of ageing is that the membrane composition of the lysosomes changes during ageing, reducing the motility of LAMP2 in the lysosomes and restricting it to microdomains[97]. This causes a reduction in the sites where the interaction between HSC70 and the cargo can occur and impairs the degradation of other CMA substrates. In an independent experiment, it was shown that the upregulation of Lamp2 during ageing improves liver function and healthspan in a transgenic mouse model[98]. In a twist, but also supporting the idea that the loss of proteostasis through CMA is deleterious and occurs in ageing, is that the upregulation of CMA components, such as LAMP2a

and HSC70, occurs in several types of tumours, such as those present in breast, colorectal, gastric, small cell lung carcinoma and Leukemia, and that blockage of CMA has proven as a therapeutic approach to reduce the size and rate of growth of the tumour[99].

There are already some known activators of autophagy that are being tested as anti-ageing drugs, such as rapamycin or metformin, that have been shown to reduce age-related inflammation, for example, spermidine, which also inhibits mTOR through the MAPK/ ERK1/2signallingg pathway, and has been observed to reduce thrombosis, age-related inflammation, or trehalose, a disaccharide that has been observed to reduce protein aggregates in neurons[100,101].

NDR2 kinase

One of these novel potential intervention sites are the nuclear nbf2-related (NDR) serine-threonine kinases (NDR). They are part of the AGC superfamily of protein kinases and core constituents of the canonical hippo pathway. Ndr1 and Ndr2, also known respectively as STK38 and STK38L (Serine-threonine kinase 38 and 38-like), act in the same fashion as their counterparts LATS1/2 (large tumour-suppressor 1/2)[102]. They were first described as upstream regulators of Yes-associated protein (YAP) and the transcriptional co-activator with PDZ binding motif (TAZ), and in turn, they are regulated upstream by Mammalian Ste20-like 1 and 2 (MST1/2), thus forming part of the core hippo pathway[103]. Besides being activated by phosphorylation by MTS1/2, it is also activated through autophosphorylation on the S282 and T442[104], and negatively regulated through protein phosphatase 2A, thus activated by phosphatase inhibitors such as okadaic acid. Upstream of this activation, Ndr2 is recruited from the cytoplasm to the plasma membrane and subsequently phosphorylated by the membrane protein Mps1 binding factor (MOB) by interacting with the NTR (N-Terminal regulatory) domain and increasing the autophosphorylation activity in the S281/282[105]. Interestingly, previous work from our group demonstrated that between the two isoforms, Ndr2 is the main NDR kinase in the adult mouse brain because the protein levels of Ndr1 rapidly decrease after birth while Ndr2 becomes more and more expressed[106].

Ndr2 in neurons

Besides the canonical activity of the hippo pathway in regulating cellular growth, it has been demonstrated that Ndr2 has a wide variety of targets and roles within the cell, particularly in neurons, as it is necessary for integrin trafficking and activation ahead of neurite growth and branching, cell

motility and migration[107]. Homologues of NDR Kinases were previously characterized in *C.elegans* (SAX-1) and *Drosophila melanogaster* (Trc: Tricornered). In the latter, it was shown that they are involved in the dendritic branching of sensory neurons because it was observed that the Trc mutants display increased branching and overlap of redundant dendrites[108]. In the former, SAX1 KD resulted in a similar dendrite overlapping phenotype[109], showing that NDR homologues are crucial for correct dendritic growth and connectivity.

Ndr2 was first identified in the mouse amygdala, and it was shown that its expression increased upon fear conditioning, suggesting a role in fear memory and learning[110]. It was later shown that overexpressing Ndr2 in forebrain excitatory neurons in adult mice increased their exploratory activity and impaired spatial memory. This was accompanied by alteration in hippocampal connectivity, synaptic transmission and altered granule cell differentiation and maturation[111], showing that it also plays a role in the hippocampus and hippocampal-dependent memory. Given the previous observations, a great effort has been put into characterizing the molecular pathways involved in cognition and where they could be involved. It was demonstrated that Ndr2 is involved in the regulation of dendritic length and dendritic arborization, as well as intracellular trafficking[112]. In hippocampal neurons, Ndr2 was identified in the soma, the dendrites and the synapses and also that its role in neurite growth is carried out through the activation and mobilization of integrins[106].

Ndr2 in cell cycle control

While not much is known about the regulation of Ndr2 during physiological ageing, a lot of links have been made between Ndr2 and some of the hallmarks of ageing, particularly in the context of cell cycle control and cellular senescence. We know that Ndr2 knockdown *in vitro* caused an increased arrest in the G_1 phase, along with an increased level of p21 (Cipi1) protein, independent of its mRNA. It was later shown that it phosphorylates p21 in S146, which is known to increase its stabilization. Whether this regulation also relates to p21 anti-apoptotic function is not known yet. It was also found that it binds to c-Myc and increases its stability.[113] Some insight for the former comes from studies where it is shown that Ndr2 KO in retinal cells from young mice increases TUNEL positive cells, as well as the activation of caspase 3; concordantly, it was shown that in retinal slices, Ndr2 KO increases proliferation. In contrast to WT slices, where the proliferation stops after postnatal day 14[114]. It is also known that the double KO of Ndr1/2 produces a developmental delay in mouse embryos starting at day E8, which culminates in the reabsorption of the product at E10.5. It was observed that mRNA of *Cdkn1a*^{Cip1} was upregulated in the double knockout embryos but without an increase in apoptotic cells, which could explain the

retardation of development. It was also shown that in the single mutant of either Ndr1 or Ndr2, p21 becomes essential for development, as it was impossible to obtain embryos carrying either Ndr deletion when p21 was Knocked down as well[115]. Also related to cell cycle progression, it is known that Cyclin D1 forms a complex with CDK4 or CDK6 that phosphorylates the retinoblastoma protein and then releases the transcription factors E2F, which are regulators of genes related to cell cycle progression through G_1/S phase, interestingly it was shown that CDK4 is able to bind to Ndr2 and enhance its activity, independently of CDK4 or CDK6, and that in this way, it reduces p21 protein levels[116].

Ndr2 in ageing

Another post-translational modification that regulates Ndr2 activity is the acetylation in K463 by Sirt1[117]. Although the role of this acetylation is still under investigation, it strongly implicates Ndr2 in lifespan regulation and ageing, as there is already plenty of evidence that Sirt1 is involved in the ageing phenomena at many different levels. Sirt1 downregulation induces cellular senescence and, conversely, prevents replicative and induced cellular senescence in several cell types, including stem-cells[118,119]. Yeast, nematodes, flies and mice overexpressing Sirt1 have increased lifespan and healthspan[120,121], which indicates that Sirt1 signalling is conserved and heavily implicated in lifespan regulation across species, even in humans there is an age-related increase in Sirt1, which correlates with maximum lifespan[122]. Although which pathways are involved in the pro-longevity effect of Sirtuin1 is still a hot topic under investigation, it is known so far that it plays a role in AMPK, Insulin growth like factor 1 (IGF-1) and mTOR signalling, nutrient sensing pathways that have been implicated in longevity and ageing regulation in mammals[123,124]. It´s not surprising in the end, that the beneficial effect of lifespan extension and anti-ageing drugs or therapies like caloric restriction, is accomplished through the upregulation of autophagy utilizing these pathways[125].

Ndr2 and integrins

Another important feature of the Ndr2 kinases is that they are highly conserved among metazoans, and in many different animal groups, they play an essential role in the development of the nervous system and the maturation of neurons. In *D. melanogaster*, the NDR kinase Trc (tricornered) regulates the formation of synapses and dendritic branching[108,126]; in *C. elegans*, SAX-1 determines the neuron shape and neurite growth[127]. The mammalian NDR family members are not the exception since they control neuronal polarity and axon guidance[128]proliferation of neuronal progenitors in the retina and are necessary for vesicular transport in amacrine cells[114]. In a previous report, it has been demonstrated that Ndr2 expression in the brain increases during postnatal development and continues well into

adulthood and that in developing neurons, it regulates dendritic growth through integrin signaling[106,129]. Integrins are heterodimeric transmembrane receptors that consist of an α and β subunit that integrate extracellular matrix (ECM) signalling with the actin cytoskeleton through adapter proteins such as vinculin, paxillin, talin, filamin and others[130]. Given their role in neuronal signal transduction, they are considered an integral mechanism that underlies synaptic plasticity. They regulate synapse biology through several pathways: integrins participate in spine development and organization via NMDAR/CaMKII dependent actin remodeling[131]. As heterodimers, they participate in LTP in a subunit-specific manner[132]. For example, the α3, α5, and α8 subunits are required for hippocampal paired-pulse facilitation (short-term synaptic plasticity), and the α3/β1 heterodimer is required for CA1 LTP[133], in contrast, β1 in coordination with β3 regulates the inhibitory LTP of GABAergic synapses in the CA1[134]. Correspondingly, the suppression of integrin signalling has strong effects on cognition: knocking down Integrin β1 in forebrain excitatory neurons impairs working memory and Knocking down α3, α5, and α8 ablates spatial memory in mice[135,136]. Supporting the evidence that integrins are crucial components of cognition in humans, it is known that several mutations in the integrins correlate to neurodevelopmental disorders from the autism spectrum by a reduction in connectivity and synaptic plasticity[137].

NDR2 in the regulation of autophagy

Concerning Ndr2 and autophagy, it was shown in human cell culture that Ndr1 is required for early autophagosome formation, positively regulating autophagy through direct interaction of Beclin1[138] independent of its kinase activity. It was also shown that Ndr1 can rescue the loss of autophagy in fly larvae with a loss of function mutation in Tricornered, the functional orthologue of human Ndr1, showing that it is a well-conserved evolutionary mechanism and suggesting that all Ndr kinases could potentially serve as autophagy regulators. It´s interesting, in this regard, that Ndr1 expression in the mice nervous system is lower or even null compared to that of Ndr2[106], contrary to other rodents like rats, in which the expression of both remains at comparable levels during postnatal development. So, the question of whether Ndr2 is also a master regulator of autophagy of particular importance in the nervous system has not been addressed so far.

HYPOTHESIS

The alteration of autophagy is an essential feature of ageing, and it has been related to many neurodegenerative diseases and even normal cognitive decline. One putative regulator of autophagy is the serine-threonine kinase Ndr2, the main NDR kinase in the mouse brain. The role of Ndr2 in autophagy regulation has not been completely addressed so far, and even less is known of this possible interaction in the context of ageing and memory. With that in mind, I decided to address the question of whether Ndr2 regulates autophagy in neurons and the adult brain and if it is involved in the memory loss that´s associated with the loss of autophagic regulation during physiological ageing.

OBJECTIVES

Main goal

To characterize the role of Ndr2 in neuronal autophagy in brain ageing and its implications in cognition.

Secondary goals

The first goal is to determine if Ndr2 participates in autophagy in neurons and the adult mouse brain and to identify pathways by which Ndr2 might regulate autophagy.

The second goal is to study if Ndr2 is involved in the impairment of autophagy and cognitive decline observed during ageing and to determine if the upregulation of autophagy during ageing can reduce the age-related cognitive decline in an Ndr2 KO mouse model.

And finally, to identify the pathways in which Ndr2 might participate in cognition in the ageing mouse brain.

METHODS

Animals

Husbandry

All experiments were performed with mice that were bred and raised in the animal facility at the Institute of Biology of the Otto-Von Guericke University. Mice were always given food and drinking water *ad libitum* and kept on an inverse 12-hour Light/dark cycle. When pertinent, animals were single-caged four weeks before behavioural experimentation. Stk38l$^{Gt(RRT116)byg}$ mice (Ndr2 KO) that were originally generated from a gene-trap clone of the ES cell line E14TG2a using gene trap, as previously described[15], were backcrossed with C57 Bl/6 BomTac mice for more than 12 generations and were maintained as a colony with regular "refreshing" of background by Bl/6 BomTac breeders.

BL/6 J mice were either maintained in-house with regular refreshing of background with BL/6 J mice or purchased at 18 months old from Jackson laboratories and kept in the animal facility until the experiments were performed. All animal housing and animal experiments were in accordance with the European regulations for animal experiments and approved by Landesverwaltungsamt Saxony-Anhalt (Permission Nr. 42502-2-1284-UniMD and Nr. 42502-2-1712-UniMD).

Timed breeding

The Ndr2 mice that were used for cell culture were obtained from homozygous Ndr2 WT or Ndr2 KO breeding pairs. Mice were put together overnight for 24 hours, the female mice were returned to their original cage, and 18.5 days after breeding, the pregnant females were anaesthetized using isoflurane and sacrificed. The embryos were collected in PBS and immediately decapitated.

Trehalose treatment

Mice that went under treatment were given drinking water supplemented with commercially available trehalose (ForMe, DE21276.) diluted in a concentration of 2.2% for a period of 10 weeks, in which weight and water consumption were monitored on a weekly basis. The water was also refreshed every week.

Glucose measurement

Blood was taken by puncturing the lateral tail vein with a sterile lancet, and circulating glucose levels were measured before and after 8 hours of fasting with an ACCU-CHECK *Guide* glucose meter.

Behavioural experiments

Morris Water Maze

The Morris Water Maze (MWM) was conducted on a circular tank with a circumference of 1.5 meters with 4 visual cues placed equidistantly from each other in the walls outside of the maze, each one represented the 4 cardinal points; north (N), south (S), west (W) or east (E), at an approximate height of 1.5 meters, so that they were visible at a 45° inclination from the point of view of the mice. The tank was filled with water at a temperature of 22°C ± 1°C and a circular platform of 10 cm in diameter was placed 15 cm away from the wall of the tank on one of the quadrants (NW, NE, SW or SE) at a depth of 1 cm from the surface. The test consisted of 5 training days, each with 6 trials, plus one probe trial with an inter-trial interval of approximately 10 minutes. On each trial, the mice were placed in the maze starting in a pseudo-random cardinal direction and left to swim freely for 1 minute or until they reached the platform; then, they were left for 20 seconds and placed back in their cages. In the case that the mice didn´t find the platform, they were gently guided to the platform and left there for a time-out period of 30 seconds. The first day consisted of a habituation period in which the platform location was changed after each trial. Starting from the second training day, the platform was fixed in the SW quadrant, and non-toxic white paint was added to the water to hide it. During the probe trial, the platform was removed. Then one week after the last day of testing, the platform was changed to the NE quadrant, and the mice underwent reversal learning for 4 days and one probe trial. The mice were recorded using a video camera fixed on the ceiling, exactly above the middle of the maze at a distance of 3 meters. The results were analyzed using the software ANYMaze.

Spontaneous alternation

Mice were placed in a Y-shaped maze with arms of 40 cm in length originating from the centre of the maze at a 120° angle from each other. Mice were left to explore freely for 10 minutes while they were being recorded. After each mouse, the maze was wiped with ethanol 10%. The number and sequence of entries to each individual arm were counted, as well as the direct and indirect entries (entering the same arm twice, and entering the previously visited arm, respectively). Every time the mouse visited

each arm once without performing direct or indirect visits, it was considered an alternation. The mice were recorded using a video camera fixed on the ceiling, exactly above the middle of the maze, at a distance of 3 meters. After each test, the maze was cleaned with 10% ethanol to remove any odours from previous mice.

Novel object recognition in the Y-maze

Mice were habituated to a Y-shaped maze with arms of 40 cm in length originating from the centre of the maze at a 120° angle from each other for a period of 8 minutes in which 2 similar objects (same shape and colour) were placed in each of two arms. Then the mice were removed from the maze, and one of those objects was replaced by a novel one (different shape and colour). After 2 minutes, mice were placed back in the maze for a period of 8 minutes. The mice were recorded using a video camera fixed on the ceiling, exactly above the middle of the maze at a distance of 3 meters, the time spent exploring each object was measured, and the discrimination index (D.I.) was calculated as follows:

$$D.I. = \frac{Time\ spent\ in\ old\ object - Time\ spent\ in\ novel\ object}{Time\ spent\ exploring}$$

After each test, the maze was cleaned with 10% ethanol to remove any odours from previous mice.

Open field

Mice were placed in a 50 cm x 50 cm square box and left to explore for 20 minutes in dim white light (35 lux). The mice were recorded using a video camera fixed on the ceiling, exactly above the middle of the maze, at a distance of 3 meters; then, using the software AnyMaze, the box was divided into a grid of 4x4 squares of 12.5 cm side each. The time spent on the corners, the rim or the center squares was measured. After each test, the maze was cleaned with ethanol 10% to remove any odours from previous mice.

Novel object recognition and location in the open field

Habituation consisted of 3 trials in which the mice were placed in a 50 cm x 50 cm square box and left to explore dim white light. At the same time, 3 different objects (different shapes and different colours) were present at a distance of 15 cm from each respective corner. The objects were made of LEGO pieces of different patterns of contrasting colours each and of different areas ranging from 3 x 3 cm to 6 x 6

cm and heights from 5 to 9 cm. For the novel location (NOL) task, the object placed in the corner most position from the free corner was relocated across the maze to the empty position at a distance of 15 cm from the free corner. Then the mice were placed back in the box. After that, mice were re-habituated to the objects for another trial. Finally, for the NOR test, one of the old objects that were not previously relocated was replaced by a new one (different shape and different colour), and then the mice were placed back in the box. Each trial lasted for 5 minutes, with an inter-trial interval of 2 minutes. The mice were recorded using a video camera fixed on the ceiling, exactly above the middle of the maze at a distance of 3 meters, the time spent exploring each object was measured, and the discrimination index (D.I.) was calculated as follows:

$$D.I. = \frac{Time\ spent\ in\ old\ objects - Time\ spent\ in\ novel\ object}{Time\ spent\ exploring}$$

After each test, the maze was cleaned with ethanol 10% to remove any odours from previous mice.

Rotarod

Mice were placed in a rotarod apparatus and the time spent until they fell from the rotor was measured. After each test, the rotarod was cleaned with ethanol 10%, and mice underwent a resting period of 2 minutes. The first training day consisted of four 60-second trials, with a constant rotation of 15 revolutions per minute (rpm). The second training day consisted of five 60-second duplicate trials with a constant speed of 15, 20, 25, 30 and 35 rpm each. The final testing day consisted of four 400-second trials with an increasing speed of 4 to 40 rpm.

Protein biochemistry

Sample preparation

Mice brains were collected, and the dorsal hippocampus, ventral hippocampus, frontal cortex, cortex and cerebellum were dissected manually. The tissue was snap frozen in liquid nitrogen and mechanically disaggregated on ice using LM Buffer (1% Laurylmaltoside, 1% NP-40, 1mM $Na_3\ VO_4$, 2mM EDTA, 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.5% DOC, 1mM AEBSF, 1uM Pepstatin A, 1mM NaF and 1 Tablet of Pierce protease inhibitor). Samples were incubated for 25 minutes at 4°C and then centrifuged at 13,000g. for 10 min at 4°C. The supernatant was collected and prepared for western blot analysis. In each case, 5µl of the protein sample was quantified using the colourimetric assay Bio-Rad Protein Assay

(BIO-RAD, 5000001), according to the manufacturer's instructions and detected using a Tecan Infinite M200 Pro microplate reader.

Western Blot

Sample lysates containing 20µg of protein were loaded in an SDS polyacrylamide gel and run at a constant voltage in running Buffer 1 X (25 mM Tris, 25 M glycine, 1% SDS) at room temperature for 100-130 V for 1,5 to 2 hours until the running front reached a height of 0.5 cm above the end of the gel. Then the proteins were transferred to an immobilon FL-PVDF membrane at a constant voltage of 120 V for 75 minutes in transfer buffer (25 mM Tris, 0.192 M glycine, 20% methanol, 10% SDS) at 4°C. Membranes were blocked by incubating them in intercept blocking solution for 1 hour. The Primary antibody solution was diluted in blocking solution with the corresponding antibodies and incubated overnight at 4°C. The membranes were washed 3 times with TBS-tween for 5 minutes and incubated in blocking solution with the corresponding secondary antibody. The protein signal was observed using near-infrared fluorometric detection and acquired using an Odyssey-scanner. Quantification and normalization of the signal were performed using the Image Studio Lite software from LICOR.

Immunohistochemistry

Brains from 3-month-old mice were dissected, and the hemispheres were split using a sterile blade. Half of the hemisphere was fixed in PFA 4% in PBS for 24 hours at 4°C. The brains were then stored in a solution of 30% Sucrose in PBS for 36 hours. Coronal brain slices of 30 µm were obtained using a cryostat and stored in PBS with 2% NaN_3. Brain slices were permeabilized with 0.3% Triton-X in PBS for 10 minutes. Antigen retrieval was performed by immersing slices in HIER buffer (10mM Tris, 1mM EDTA, 0,05% Tween-20) and incubating them at 95°C for 30 minutes. Slides were washed with PBS 3 times and then incubated in blocking solution (PBS with 2% Donkey serum or BSA) for 1 hour at room temperature. Primary antibodies were incubated in blocking overnight; then, the slides were washed with PBS 3 times and incubated in blocking solution containing the appropriate secondary antibodies for 1 hour at room temperature. Slides were air-dried in glass slides before mounting them in fluoromount with DAPI.

Cell culture and Immunocytochemistry

Primary neurons were obtained from fetal hippocampus of mice at E18.5. Cells were seeded in DMEM supplemented with 10% Fetal bovine serum, 1% glutamax, and 1% penicillin/streptavidin at a density of

approximately $2.6x10^5$ cells per cm^2 over coverslips coated with Poly D-Lysine. After 24 hours of incubation, media was removed, and NB Media supplemented with conditioned media (1:1) and 2% B27, 1% glutamax and 1% penicillin/streptavidin was added. After 14 DIV, cells were washed with warm $HBSS^{+/+}$; then they were fixed using PFA 4% in PBS, permeabilized with 0.3% Triton-X in PBS for 10 minutes, and then blocked for 1 hour in blocking solution (PBS, 5% Bovine serum albumin, donkey serum or goat serum) at room temperature. Cells were incubated in blocking solution with primary antibody overnight at 4°C, then washed 3 times with PBS and incubated in blocking solution with the corresponding secondary antibody.

Mass spectrometry

The proteins were identified using a tri-hybrid linear ion-trap quadrupole-Orbitrap mass spectrometer, Fusion Lumos ETD. The results were analyzed with the Orbitrap mass analyzer. To filter the data, only the proteins that were quantified with two unique peptide matches were kept for the data analysis. Moreover, only proteins were kept if they were quantified in at least 2 out of the 3 technical replicates. To transform the data for quantification, the vsn package from Wolfgang Huber was used to apply a variance stabilization normalization method on the log2 raw data. A protein was considered a “hit” if the false discovery rate was smaller than 0.05 and had a fold change of at least 2. On the other hand, a protein was considered a “candidate” if the false discovery rate was smaller than 0.5 and had a fold change of at least 1.5 when compared to the control group when the comparison was performed.

Data Analysis

Navigation Strategy Analysis

To analyze the navigation strategies used by the mice in the MWM, the open-access Python script developed by Mathew Cooke, "Pathfinder: open source software for analyzing spatial navigation search strategies", was used. The x and y coordinates were obtained from the software AnyMaze, and the strategies were defined as follows:

Direct Path	
Ideal Path Error Maximum	150
Heading Error (max, degrees)	45
Focal Search	
Distance to centroid (max, % of radius)	40
Distance to goal (max, % of radius	50
Distance covered (min, cm)	1
Distance covered (max, cm)	300
Directed search	
Time in angular corridor (max, % of trial)	51
Distance covered (max, cm)	900
IPE Maximum	300
Indirect search	
Ideal Path Error Maximum	900
Average heading error (max)	70

Chaining	
Time in annulus zone (max % of maze)	51
Quadrants visited (min)	4
Area of maze traversed (max % of maze)	80
Scanning	
Area of maze traversed (max % of maze)	25
Area of maze traversed (min % of maze)	10
Average distance to maze centre (max % of radius)	40
Random search	
Area of maze traversed (min % of maze)	25
Thigmotaxis	
Time in full thigmotaxis zone (min % of trial)	50
Time in full thigmotaxis zone (max % of trial)	50
Total distance covered (min, cm)	450

*Uncategorized trials were classified manually by a blind observer.

Microscopy

All images were acquired using a Leica thunder microscope (Cat No, Leica, DMi8) equipped with a CDD camera (Cat. No. MC170 HD, Leica). All pictures of primary neurons were taken using a 63x oil immersion apochromatic objective (Cat no. 506192). Each picture consisted of at least 14 pictures in a z stack of 4 µM. The pictures of dorsal hippocampus slices were taken using a 20x multipurpose fluotar objective (Cat no. 11556068). Image acquisition, deconvolution and merging were performed using the LASX software from Leica.

Image analysis

Images were analyzed using the one software Icy. The puncta analysis was performed using the built-in spot detector. When relevant, ROIS were drawn manually. The colocalization analysis was performed using the "synapse colocalizer" protocol from Icy. The relative intensity was obtained from the same software.

Statistical analysis

All statistical analyses and graphs were produced with GraphPad PRISM 9.1. The following statistical tests were performed depending on the data analyzed: Pairwise comparisons were evaluated with a two-tailed paired Student´s t-test. When two conditions were present, a Two-way ANOVA with Fisher´s LSD test, Two-way RM ANOVA with Bonferroni´s posthoc comparison (immunohistochemistry and biophysical measurements) or Two-way ANOVA with Tukey´s posthoc comparison (behavioural analysis) was used. In the case that more than two factors were evaluated, a Three-way ANOVA with Tukey´s posthoc comparison was performed (e.g. Treatment, age and genotype). Regardless of the test, the statistical threshold for significance was always <0.05. The test and the significance value for each comparison are further explained for each figure.

Antibody list

Primary antibodies

Antibody	Concentration (WB)	Concentration (IHC/ICC)	Brand, and Cat. No.	Species reactivity	Incubation time
LC3 A/B	1:2,000	1:400	Cell signaling,12741S	Rabbit	overnight
P62	1:1,000	1:400	Cell Signaling, 5114	Rabbit	overnight
TFEB	1:1,000	1:400	Bethyl, A303	Rabbit	overnight
Lamp I	1:1,000		Protein tech, 21997	Rabbit	overnight
α Tubulin	1:20,000		Sigma-Aldrich, T6199	Mouse	1 hour
MAP2		1:500	Synaptic Systems, 188004	Guinea Pig	overnight
Integrin β1		1:200	Abcam, ab5189	Rabbit	overnight
Drebrin-1		1:5,000	MBL, D029-3	Mouse	overnight
Galectin 1		1:400	Santa Cruz, sc-23938	Rat	overnight
ApoE		1:400	Cell Signaling, 68587	Rabbit	overnight

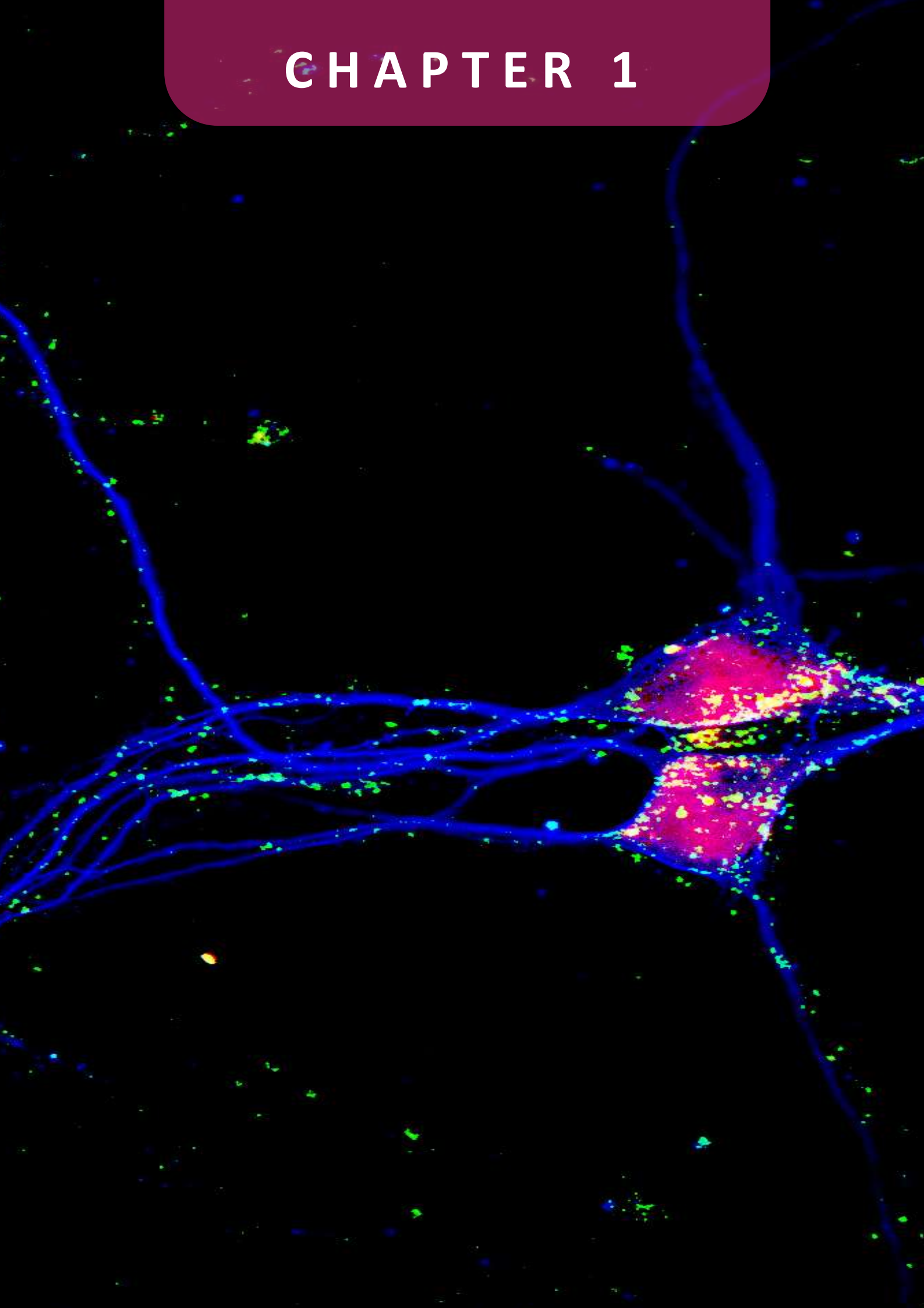
Secondary antibodies

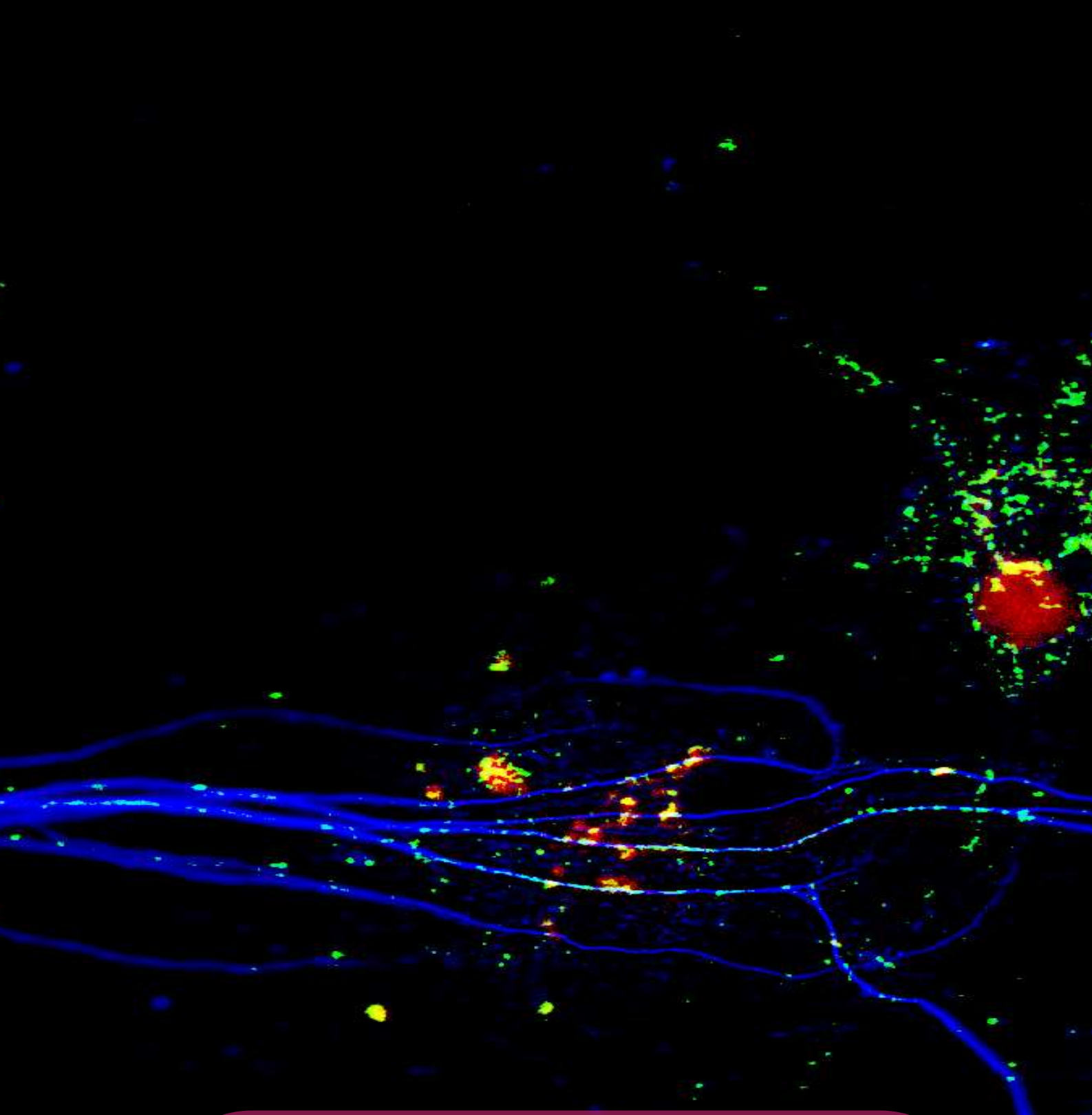
Antibody	Concentration (WB)	Concentration (IHC/ICC)	Brand, and Cat. No.	Species reactivity	Incubation time
IR 680 mouse	1:20,000		LICOR, 925-32211	Goat	1 hour
IR 800 rabbit	1:20,000		LICOR, 926-68070	Goat	1 hour
Alexa fluor 488		1:1,000	Invitrogen A21202	Goat	1 hour
Alexa fluor 488		1:1,000	Invitrogen A11055	Mouse	1 hour
Alexa fluor 644		1:1,000	Invitrogen A21450	Guinea Pig	1 hour
Alexa fluor 555		1:1,000	Invitrogen A21432	Rabbit	1 hour
Alexa Fluor 647		1:1,000	Invitrogen A21247	Rat	1 hour

Reagent list

Reagent	Catalogue No.
Acrylamide 30%	Carl Roth, A124,1
B27	Gibco, 17504044
Bovine serum albumin	Merck, A9418
DMEM	Gibco, 31885049
Donkey Serum	Sigma, S2170-100
Fluoromount	Thermo, 00-4959-52
Glutamax	Gibco, 35050061
Goat Serum	Sigma, S2000-100
HBSS-/-	Gibco, 14175095
HBSS+/+	Gibco, 14025092
Intercept Blocking buffer	Li-Cor, 927-700001
Isoflurane	Baxter, HDG9623
Neurobasal	Gibco, 10888022
PFA	Roth, 0335.1
Poly D-Lysine	Gibco, A3890401
Protease & phosphatase inhibitor	Thermo, 13393126
Protein Assay	BIO-RAD, 5000001
PVDF membranes	Immobilion, IPFL000010
Triton X	Sigma, X100
Tween 20	Roth, 9127.1

CHAPTER 1





NDR2 REGULATES AUTOPHAGY IN HIPPOCAMPAL NEURONS AND IN THE DORSAL HIPPOCAMPUS

Ndr2 deficiency increases autophagosomes in the synapses of hippocampal neurons

In order to determine if Ndr2 plays a role in neuronal autophagy, a hippocampal neuronal culture model in which autophagy could be induced was first established. Hippocampal neurons from Ndr2 WT and . pups taken at embryonic day 18.5 and after 2 days in vitro (*DIV*) were treated with cytarabine, also commonly known as Arabinoside C (Ara C), a commonly used drug that binds to DNA and stops replication, used to reduce the amount of non-neuronal cells in the final culture (Figure 1). After 14 DIV cells were fixed, the neurons were immunostained with MAP2 (green) and the glial cells

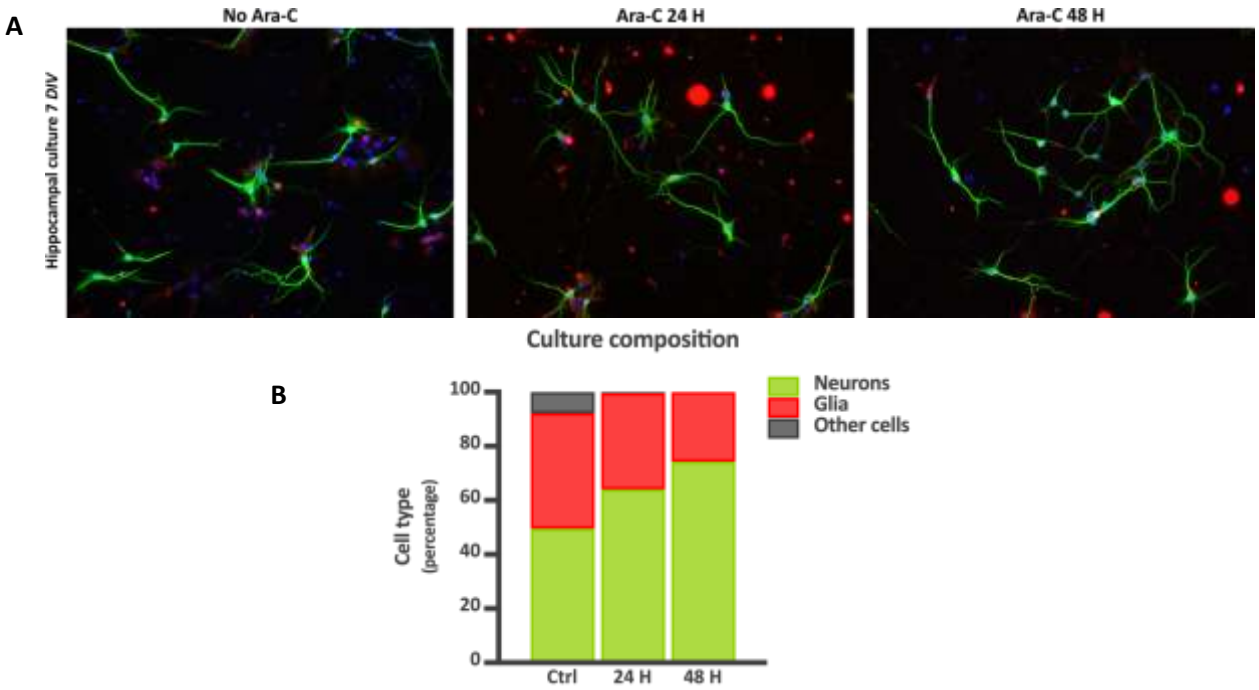


Figure 1. Ara C treatment reduces the number of proliferating cells in a hippocampal cell culture after 14 DIV. Hippocampal cells taken from pups at E18.5 were grown in a PDL coated coverslip for 48 hours. Then cells were treated with Ara C 3µM for 24 or 48 hours. Media was removed and cells were left until they reached DIV 14. Cells were fixed and immunocytochemistry was performed against MAP2, or GFAP (A). Cells were quantified (B) and classified either as neurons (green) glial cells (red) or other cells) no staining.

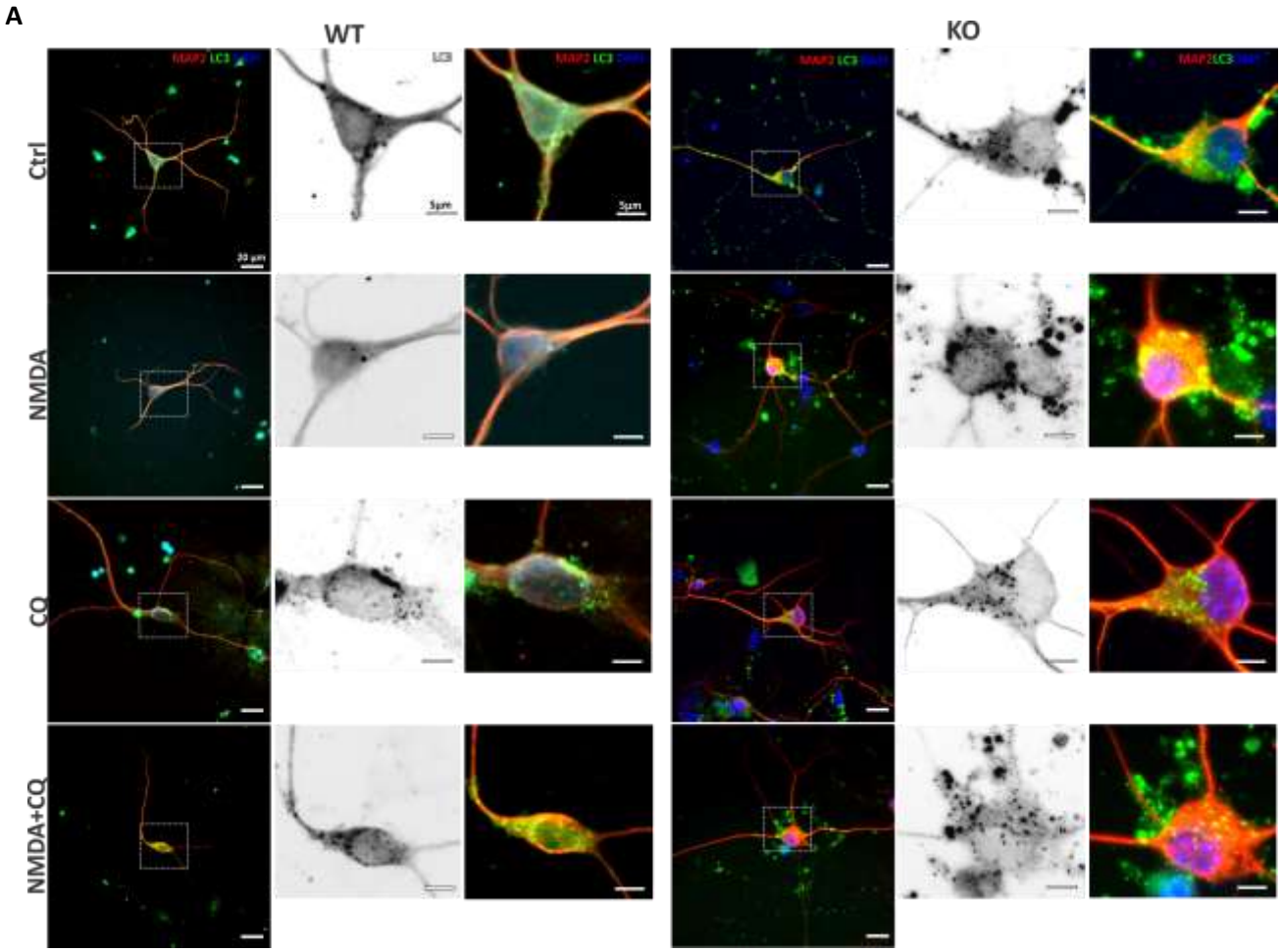
with GFAP (red). The composition of the culture treated with Ara C for 48 hours showed a higher proportion of neurons compared to glial cells and no cells of unidentified cell types.

Figure 2. Induction of autophagy in hippocampal neurons. Mice embryos were taken at embryonic day 18.5, and the hippocampus was dissected. Hippocampal primary neuronal culture was prepared. Neurons at DIV 14 were either treated with NB medium supplemented with NMDA 50µM, Chloroquine 50µM, or NMDA + Chloroquine for 2 hours.

Afterwards, a model for autophagic flux induction in hippocampal neurons was established. Previous reports showed that hippocampal neurons treated with a combination of NMDA and Chloroquine (CQ) showed an increase in the autophagic flux that reached its peak after 2 hours[139], which relates to the role of autophagy in degradation of the AMPA receptors[64] during long-term depression (LTD). With that in mind, hippocampal neurons were treated with NMDA 50 µM, CQ 50 µM, or NMDA +CQ in NB media. After 2 hours of treatment, neurons were washed and fixed in PFA 4% (Figure 2). Autophagic flux was evaluated by immunochemistry of LC3 (Figure 3) and p62 (Figure 4).

We observed that the NMDA, CQ or the combined treatment of NMDA+CQ induced the accumulation of LC3 puncta in the WT and KO neurons and also that the KO neurons showed an increase of LC3 puncta regardless of treatment compared to the WT neurons. Supporting what has been published before, the treatment with NMDA + CQ increased the p62 puncta, regardless of the genotype. This suggests that the absence of Ndr2 might increase autophagosome formation and that these autophagosomes might not necessarily be related to an alteration in the basal degradative autophagy. Thus, to analyze the possibility that Ndr2 might alter the localization and possibly the transport of autophagosomes within the neuron, I analyzed the localization of the LC3 puncta in different neuronal compartments (Figure 5). The neurons were divided into 4 different regions of interest (ROI s), one corresponding to the soma (blue), one to the primary dendrites (purple), one for the secondary dendrites (orange), and finally, one for the axon yellow). The LC3 puncta distribution in the different ROIs was calculated for each individual neuron. We observed that in basal conditions, KO neurons have fewer LC3 puncta in the primary dendrites but more in the secondary and that after NMDA treatment, the percentage of autophagosomes in the soma increased compared to the WT neurons. Given that the spines, and consequently the synapses, are located in the dendrites, I thought of the possibility that the alteration in the autophagic flux observed in the KO neurons might have implications in synaptic autophagy and possibly plasticity. With that in mind, I decided to analyze if the overall effect observed in the autophagosome accumulation in the Ndr2 KO hippocampal neurons also occurred at the synapses. *DIV*

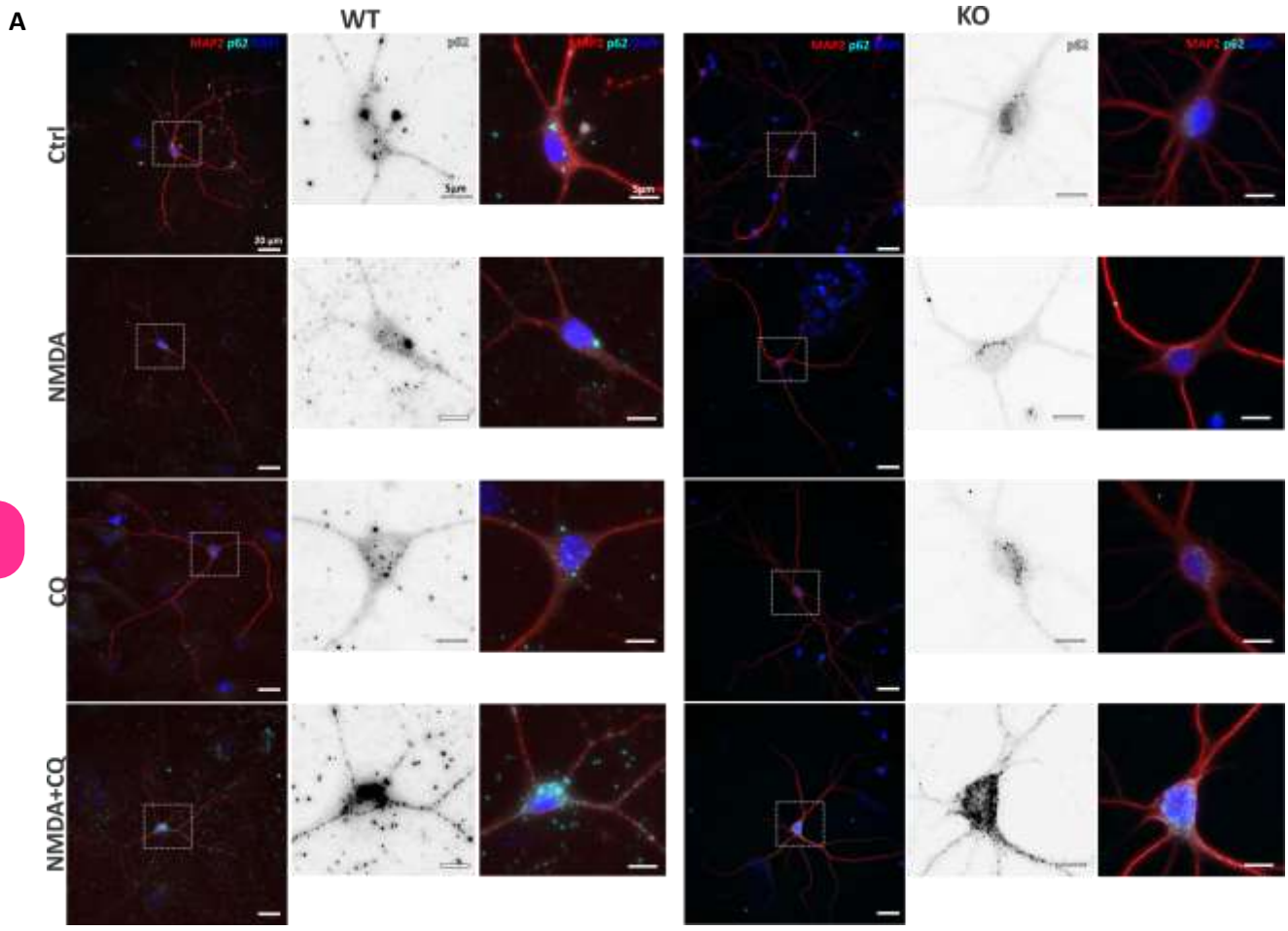
14 Hippocampal neurons treated with NMDA, CQ or both for 2 hours were immunostained with phalloidin, LC3 and Drebrin, a postsynaptic actin-binding protein required for spine formation that is transported to the dendritic spines upon activation of the NMDA receptors[140]. We analyzed the colocalization of the LC3 puncta with Drebrin in secondary dendrite segments of 50µm in length (Figure 6). Contrary to what I expected, I didn´t observe any changes in the autophagosome distribution in the synapses upon induction of autophagy in the WT or KO neurons; nonetheless, I observed that Ndr2 KO hippocampal neurons upon treatment with NMDA, but not chloroquine, consistent with our previous results.

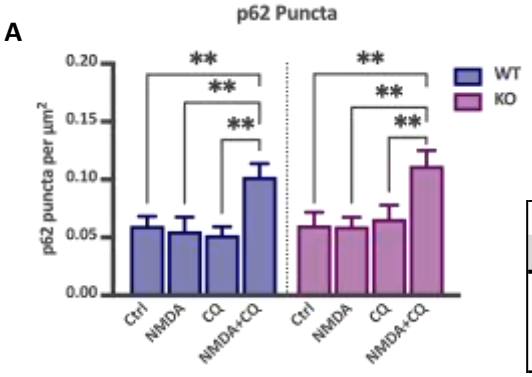


C

LC3 Puncta			
Source of variation	P value		F (DFn, DFd)
Interaction	5,40%	0,0638 ns	F (3, 73) = 2,530
Genotype	9,86%	0,0052 **	F (3, 73) = 4,615
Treatment	30,29%	<0,0001 ****	F (1, 73) = 42,55

Figure 3. Ndr2 KO Hippocampal neurons have increased autophagosome density. Hippocampal cells taken at E18.5 were treated with NMDA 50µM, Chloroquine 50µM, or both for 2 hours after 14 *DIV*. Immunocytochemistry against LC3 and MAP2 was performed (A), and LC3 puncta (green) in hippocampal neurons (red) were quantified using the software icy (B). The bars represent the mean+ S.E.M. *Two-way ANOVA;* *p<0.05.; **p<0.005 n=10 neurons.



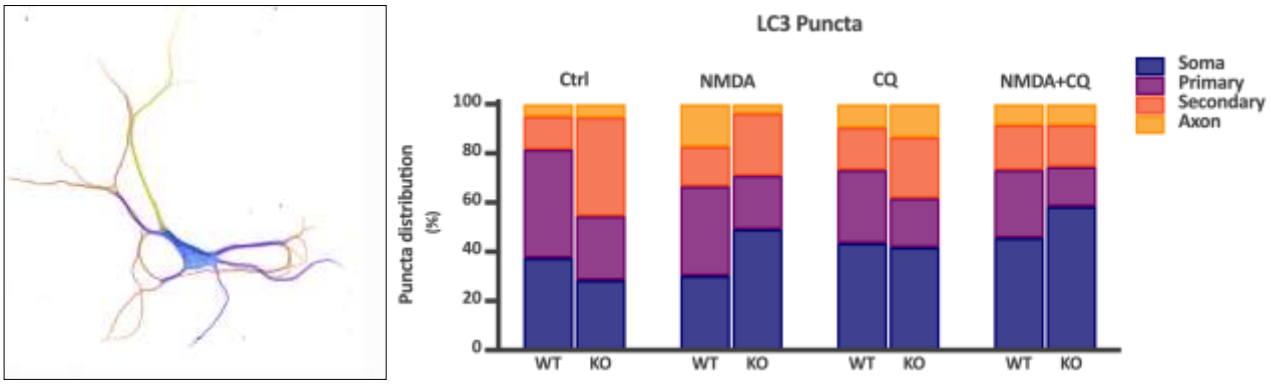


B

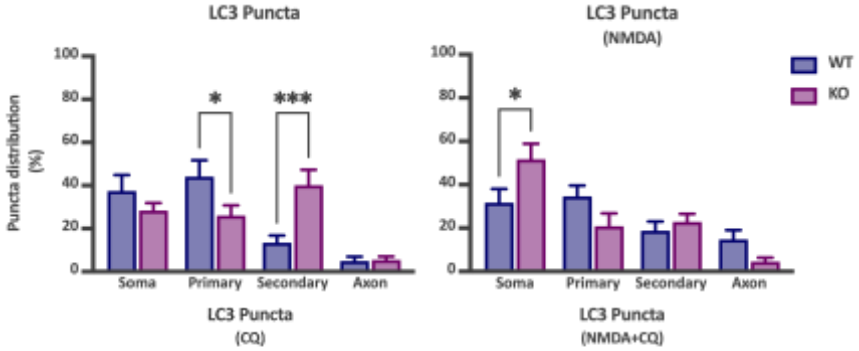
p62 Puncta			
Source of variation	P value		F (DFn, DFd)
Interaction	0,41%	0,9321 ns	F (3, 75) = 0,1457
Treatment	27,88%	<0,0001 ****	F (3, 75) = 9,809
Genotype	0,79%	0,3637 ns	F (1, 75) = 0,8351

Figure 4. KO Hippocampal neurons show no alteration in the p62 puncta accumulation upon induction of autophagic flux. Hippocampal cells taken at E18.5 were treated with NMDA 50µM, Chloroquine 50µM, or both for 2 hours after 14 DIV. Immunocytochemistry against p62 and MAP2 was performed (A), and p62 puncta (cyan) in hippocampal neurons (red) were quantified using the software icy (B). The bars represent the mean+ S.E.M. Two-way ANOVA; *p<0.05.; **p<0.005 n=10 neurons.

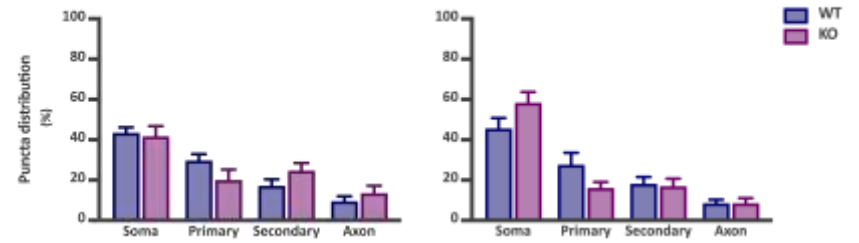
A



B



C



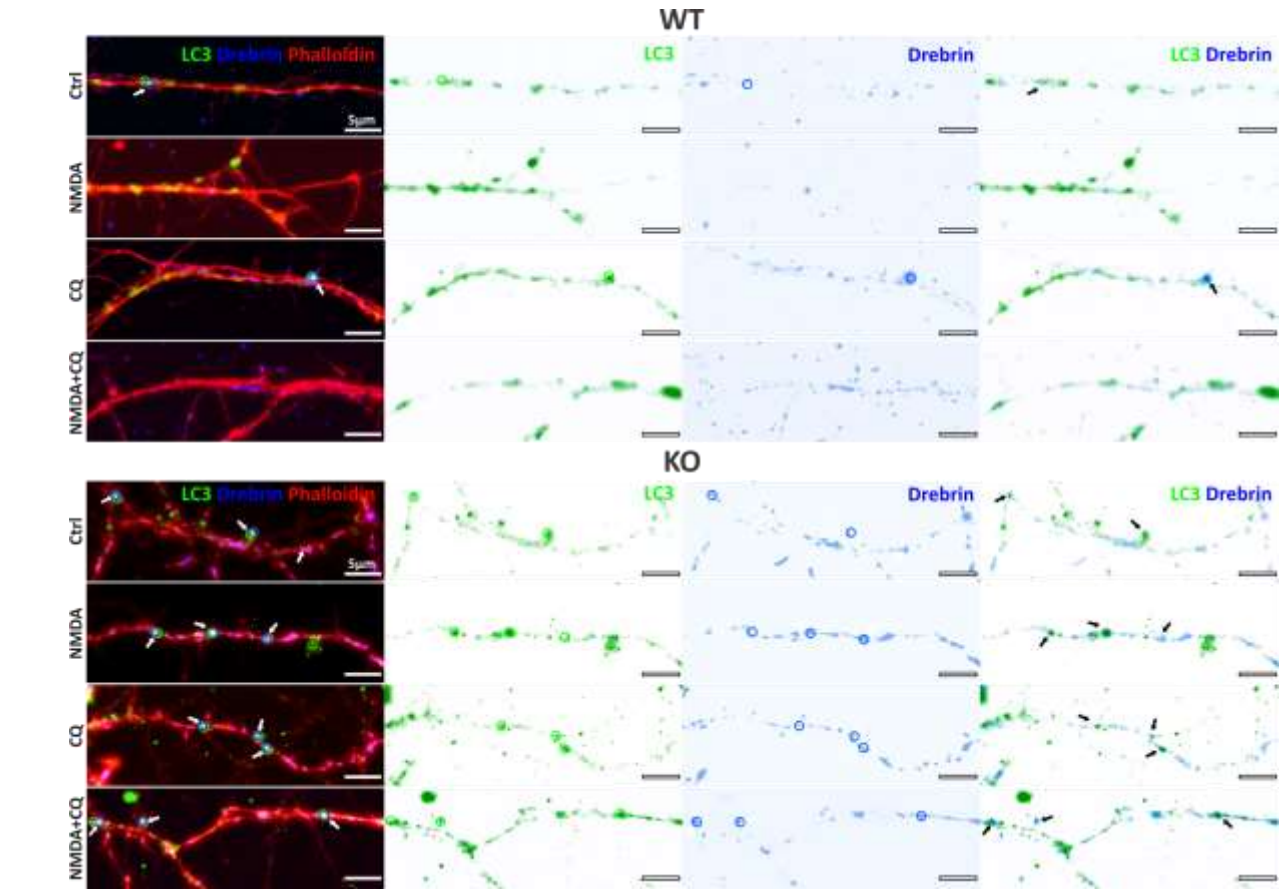
LC3 Puncta distribution-Ctrl			
Source of variation		P value	F (DFn, DFd)
Region x Genotype	14,77%	0,0034 **	F (3, 57) = 5,085
Genotype	29,41%	<0,0001 ****	F (3, 57) = 10,13
Region	0,00%	0,4096 ns	F (1, 19) = 0,7109
Mice	0,00%	>0,9999 ns	F (19, 57) = 1,913e-006

LC3 Puncta distribution-NMDA			
Source of variation		P value	F (DFn, DFd)
Region x Genotype	4,24%	0,0974 ns	F (3, 54) = 2,210
Region	61,19%	<0,0001 ****	F (3, 54) = 31,87
Genotype	0,00%	>0,9999 ns	F (1, 18) = 0,000
Mice	0,00%	>0,9999 ns	F (18, 54) = 3,618e-008

LC3 Puncta distribution-NMDA+CQ			
Source of variation		P value	F (DFn, DFd)
Region x Genotype	4,24%	0,0974 ns	F (3, 54) = 2,210
Region	61,19%	<0,0001 ****	F (3, 54) = 31,87
Genotype	0,00%	>0,9999 ns	F (1, 18) = 0,000
Mice	0,00%	>0,9999 ns	F (18, 54) = 3,618e-008

LC3 Puncta distribution-CQ			
Source of variation		P value	F (DFn, DFd)
Region x Genotype	4,24%	0,0974 ns	F (3, 54) = 2,210
Region	61,19%	<0,0001 ****	F (3, 54) = 31,87
Genotype	0,00%	>0,9999 ns	F (1, 18) = 0,000
Mice	0,00%	>0,9999 ns	F (18, 54) = 3,618e-008

Figure 5. Ndr2 KO Hippocampal neurons show altered autophagosome distribution in basal conditions and upon treatment with NMDA. Hippocampal cells taken at E18.5 were treated with NMDA 50µM, Chloroquine 50µM, or both for 2 hours after 14 DIV. Immunocytochemistry against LC3 and MAP2 was performed and LC3 puncta in hippocampal neurons were quantified using the software Icy. The neuron was divided into four different ROIs corresponding to the Soma (blue), primary (purple), secondary (blue) dendrites and the axon (yellow) (A). The puncta distribution on each condition and each ROI was quantified (B). The bars represent the mean + S.E.M. Two-way RM ANOVA with Bonferroni post-hoc comparison; *p<0.05.; **p<0.005 n=10 neurons (C).

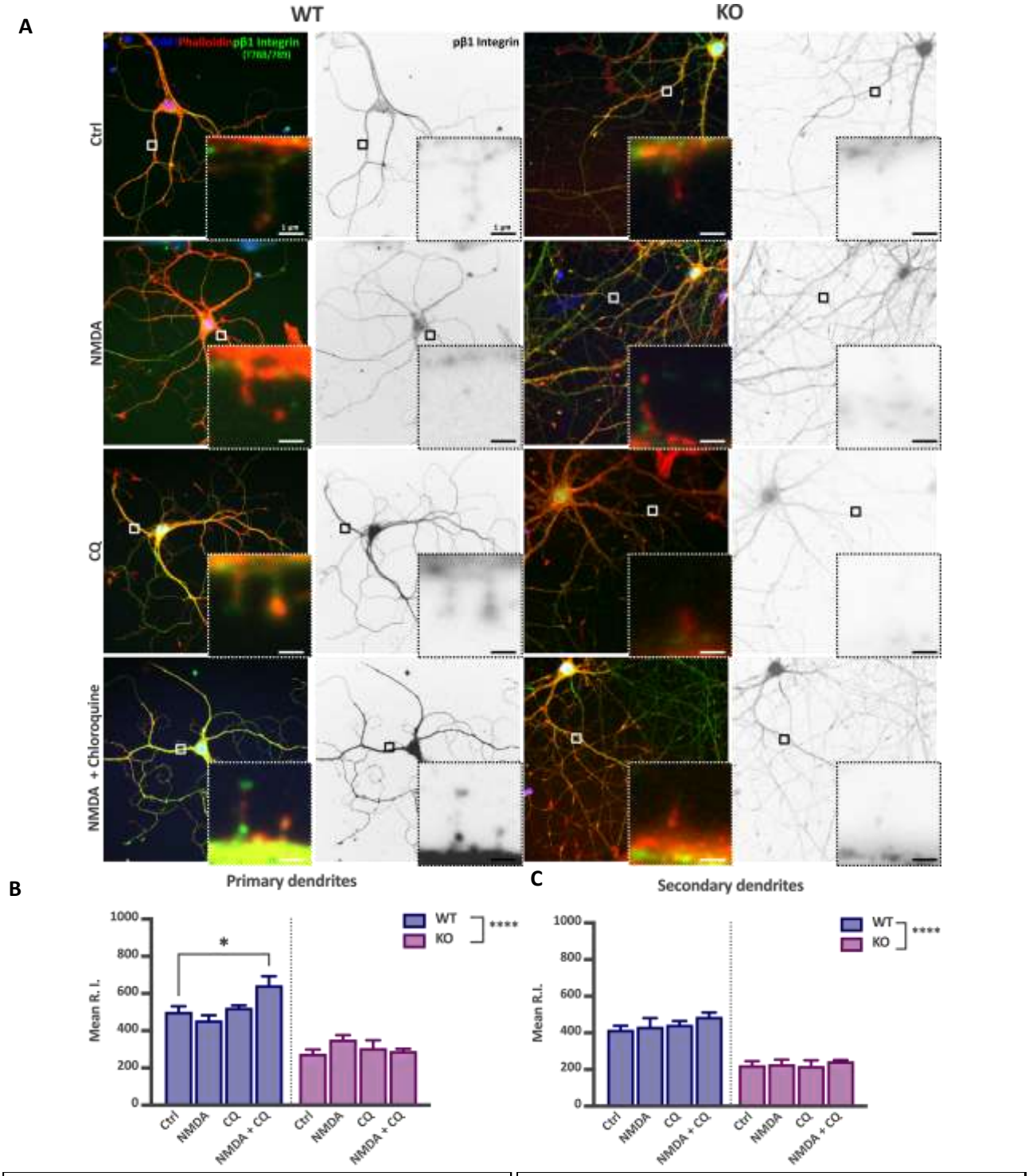


LC3 in spines			
Source of variation	P value		F (DFn, DFd)
Interaction	1,71%	0,561 ns	F (3, 85) = 0,6895
Treatment	0,65%	0,8505 ns	F (3, 85) = 0,2649
Genotype	25,78%	<0,0001 ****	F (1, 85) = 31,28

Figure 6. Ndr2 KO Hippocampal neurons show increased autophagosome numbers in the synapses. Hippocampal cells taken at E18.5 were treated with NMDA 50µM, Chloroquine 50µM, or both for 2 hours after 14 DIV. Immunocytochemistry against LC3 and Drebrin was performed, then cells were immunostained with Rhodamine Phalloidin (A). The colocalization of all the LC3 puncta and Drebrin in a 50µm dendrite segment of the secondary dendrites was calculated with the colocalizer protocol of the software Icy. (B) The bars represent the LC3 puncta that colocalize with Drebrin in 2 dendrite segments of each neuron +S.E.M. Two-way ANOVA; *p<0.05.; ****p<0.00005 n=10 neurons.

Ndr2 deficiency decreases Integrin β1 signalling in the dendritic spines.

A very important feature of autophagy is the regulation of plasma membrane proteins, either by direct degradation or by non-conventional autophagic secretion, which has recently become of particular interest to the biology of ageing, given the involvement of degradative and secretory autophagy in age-related diseases[141] . An interesting downstream target of autophagy regulation are the integrins, which are intracellular receptors that bind to extracellular matrix proteins and lipids to integrate signals mainly related to migration, adhesion and cell-to-cell communication. In neurons, which are cells that do not usually migrate, integrins acquire an essential role in plasticity and memory formation. Interestingly, NDR2 has been implicated in the phosphorylation and subsequent activation of the Integrin β1 at the threonine 788 and 789[106]. Given that I observed changes in the autophagosome distribution in the KO neurons, I evaluated the possibility that the integrin signalling could be compromised as well. Ndr2 WT and HO hippocampal neurons were immunostained against the phosphorylated (T788/89) Integrin β1 subunit (p-β1) and phalloidin; the intensity of p- β1 in dendritic spines of both primary and secondary dendrites was evaluated (Figure 7).



Primary dendrites			
Source of variation		P value	F (DFn, DFd)
Interaction	7,92%	0,0018 **	F (3, 69) = 5,545
Treatment	3,77%	0,0565 ns	F (3, 69) = 2,637
Genotype	52,98%	<0,0001 ****	F (1, 69) = 111,3

Secondary dendrites			
Source of variation		P value	F (DFn, DFd)
Interaction	0,51%	0,7787 ns	F (3, 69) = 0,3646
Treatment	1,75%	0,2967 ns	F (3, 69) = 1,255
Genotype	65,11%	<0,0001 ****	F (1, 69) = 140,1

Figure 7. Ndr2 KO neurons have decreased Integrin β1 subunit activation, that doesn´t change upon autophagy modulation. Hippocampal cells taken at E18.5 were treated with NMDA 50μM, Chloroquine 50μM, or both for 2 hours after 14 *DIV*. Immunocytochemistry against phosphorylated T788/789 Integrin β1 subunit (green) and MAP2 (red) was performed (A), the intensity of phospho-Integrin β1 in all the spines in a 50μm dendrite segment of the primary (B) and secondary (C) dendrites was quantified. The bars represent the mean intensity of the spines in 3 dendrite segments of each neuron +S.E.M. *Two-way ANOVA;* *p<0.05.; ****p<0.00005 n=10 neurons.

Ndr2 deficiency increases LC3 protein levels and decreases integrin β1 activation in the dorsal hippocampus

In the WT neurons, I observed an increased accumulation of p-Integrin β1 that corresponded with the accumulation of autophagosomes upon treatment with NMDA+CQ in the primary dendrite spines but not in the secondary. On the other hand, I observed a decrease in the p-Integrin β1 signal in the KO

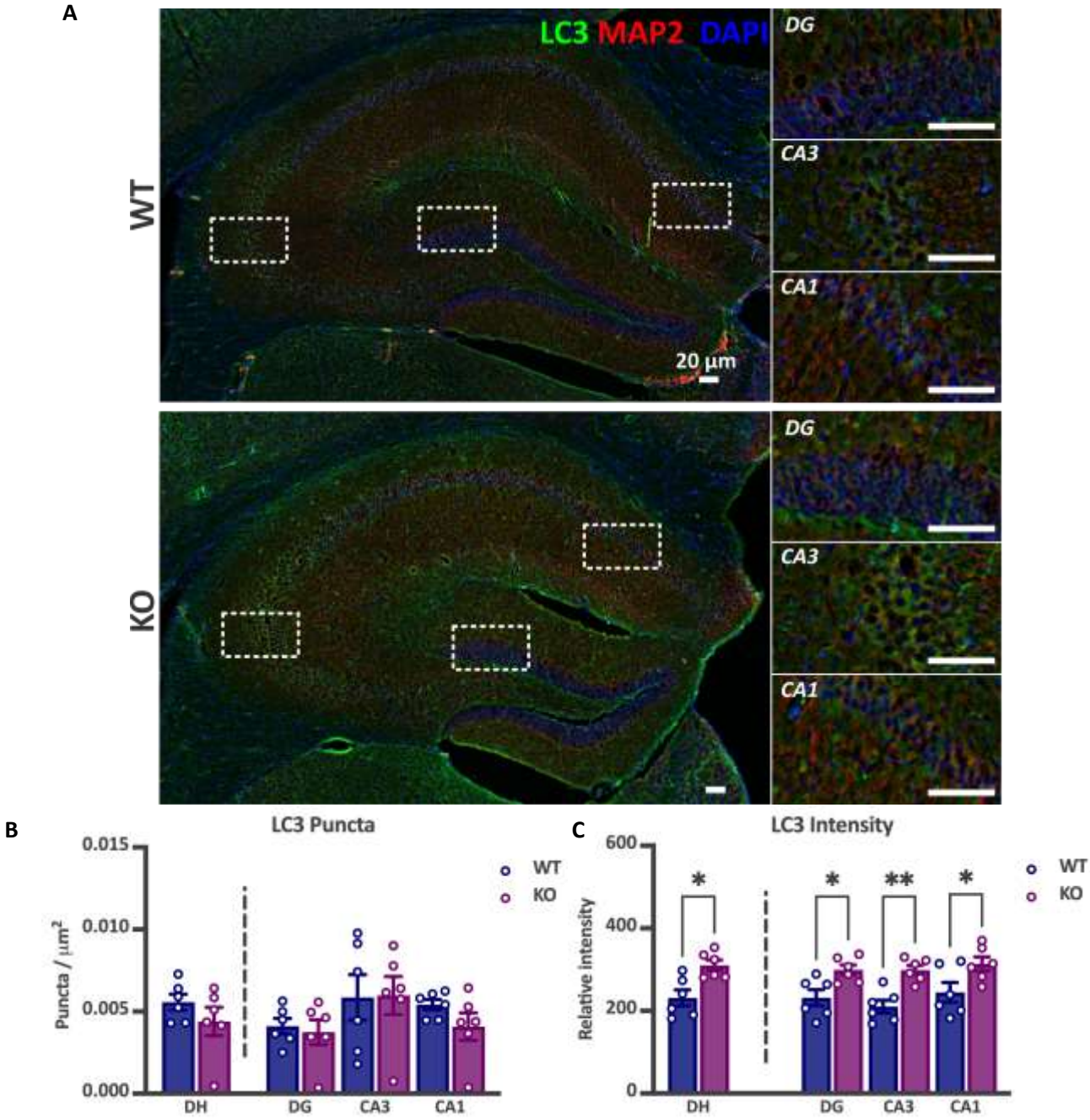


Figure 8. Ndr2 KO Mice have increased LC3 intensity in the dorsal hippocampus. 30µm Dorsal hippocampus brain slices taken from 3-month-old Ndr2 WT and KO mice were immunostained against LC3 A/B (green) and MAP2 (red) (A). LC3 puncta (B) and LC3 intensity (C) in the DH and in the DG, CA1 and CA3 brain regions were quantified using the spot detector from the software Icy. The bars represent the mean ± S.E.M. Each dot represents the average of 2 brain slices taken from the same mice. Two-tailed unpaired Student´s t-test, *p<0.05.; **p<0.005. n=6.

spines that wasn't changed upon treatment either with NMDA or CQ. In order to investigate if the observations of cultured neurons *in vitro* carried out to the adult brain, I analyzed the basal autophagic flux of 3-month-old Ndr2 WT and KO mice. First, I performed immunohistochemistry against LC3 A/B and MAP2 in 30µm hippocampal brain slices to analyze the autophagosome accumulation (Figure 8). The dorsal hippocampus was divided into 3 ROIs corresponding to the dentate gyrus (DG), and the Cornu Ammonis 1 and 3 (CA1 and CA3, respectively) and the LC3 puncta were quantified in each ROI. Even though I didn´t observe any significant differences in the LC3 puncta accumulation in the DH, or when evaluated region by region, I could observe a significant increase in the LC3 staining intensity in the KO DH that occurred evenly across all brain DH regions. To confirm these observations, I performed a Western blot (WB) analysis of DH samples of 3-month-old WT and KO mice (Figure 9). We also observed an increase in total LC3 protein levels in the KO mice compared to the WT mice.

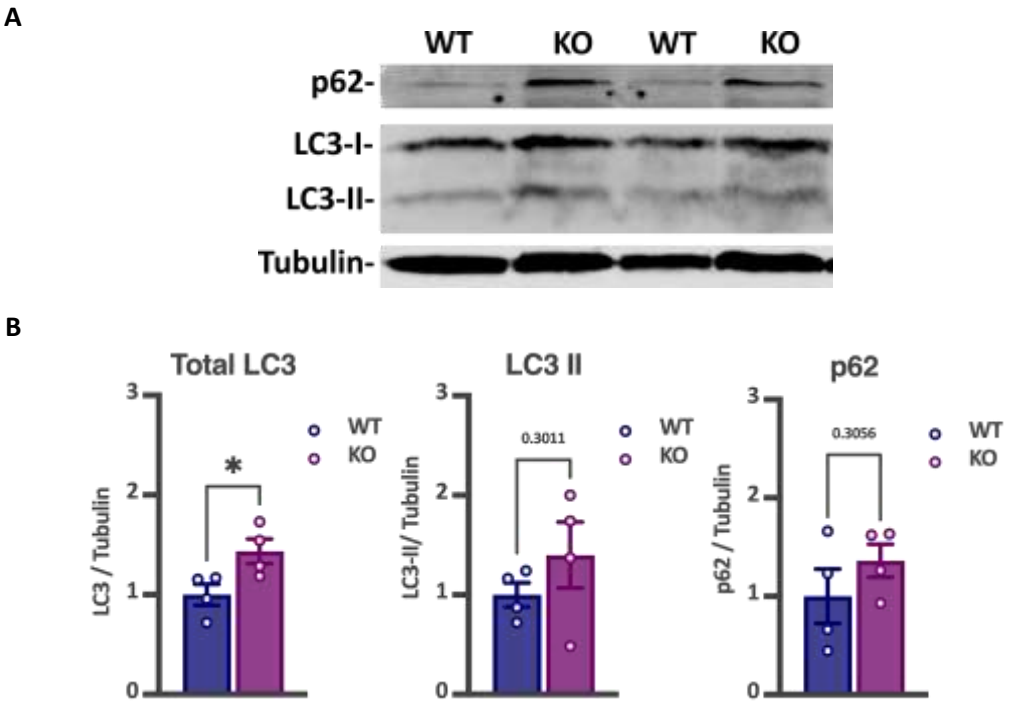


Figure 9. Ndr2 KO Mice have increased LC3 protein levels in the dorsal hippocampus. WB analysis was performed with dorsal hippocampus brain samples taken from 3-month-old Ndr2 WT and KO mice against LC3 and p62 (A). Total LC3, as well as LC3-II and p62 protein levels were quantified (B). The bars represent the mean ± S.E.M. Each dot represents the DH of each mice. Two-tailed unpaired Student´s t-test, *p<0.05. n=4.

Finally, to determine if the decrease of the activated p-Integrin β1 also occurred in the adult hippocampus, immunohistochemistry against the phosphorylated (T788/89) Integrin β1 subunit and MAP2 was performed in DH brain slices (Figure 10). We observed a decrease in the p-β1 signal in the DH. When analyzing the different hippocampal areas, I found that the decrease was more evident in the DG and CA1, but not the CA3, where I only observed a trend to decrease that was not significant.

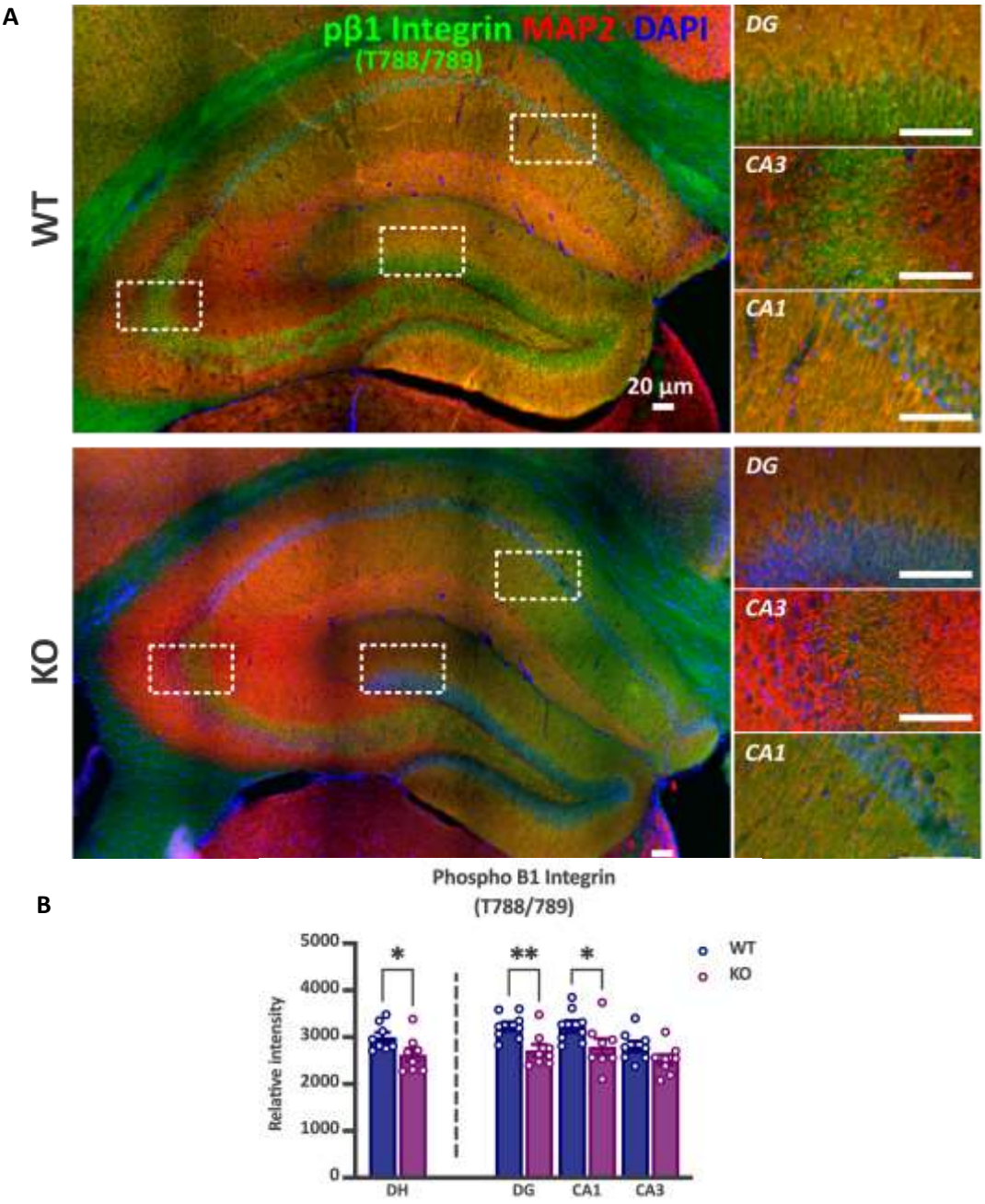


Figure 10. Ndr2 KO Mice have decreased Integrin β1 subunit activation in the dorsal hippocampus, that is more evident in the dentate gyrus and the CA1. 30µm dorsal hippocampus brain slices taken from 3-month-old Ndr2 WT and KO mice were immuno-stained against phosphorylated T788/789 Integrin β1 subunit (green) and MAP2 (red) (A), the intensity of phospho-Integrin β1 in the DH and in the DG, CA1 and CA3 brain regions were quantified using the software icy (B). The bars represent the mean ± S.E.M. Each dot represents the one brain slice. Two-tailed unpaired Student´s t-test; *p<0.05.; **p<0.005. n=8

Discussion

Summarizing the main findings presented in this chapter, I established a protocol in which autophagy could be induced with NNMDA in primary hippocampal neurons of Ndr2 WT and KO mice. I observed that Ndr2 KO neurons had increased autophagosome formation compared to the WT neurons, regardless of whether autophagy was increased with NMDA, degradation was blocked with CQ or both. Also, that p62 puncta accumulated upon treatment with NMDA and CQ, regardless of the genotype. I demonstrated that the localization of the autophagosomes in the dendrites of the Ndr2 KO neurons was altered in basal conditions and upon treatment with NMDA and that Ndr2 KO neurons had more autophagosomes in the synapses. I also demonstrated that the KO neurons have decreased phosphorylated integrin β1 and that its phosphorylation didn´t change when neurons were treated with NMDA + CQ. These observations were confirmed in vivo; Adult Ndr2 KO mice had more LC3 puncta in the DG, CA3 and CA1 hippocampal regions, increased LC3 II and p62 protein levels, as well as decreased phosphorylated integrin β1 in the DG and CA1. These results suggest that Ndr2 positively participates in autophagy by controlling autophagosome formation and autophagosome transport within the neurons.

The NMDA, CQ or the combined treatment of NMDA+CQ induced the accumulation of LC3 puncta in the WT and KO neurons as was previously described. Still, surprisingly, the KO neurons showed an increase of LC3 puncta regardless of treatment compared to the WT neurons, in a similar fashion to what was expected from the literature, the treatment with NMDA + CQ increased the p62 puncta, regardless of the genotype. This suggests that the absence of Ndr2 might increase autophagosome formation and that these autophagosomes might not necessarily be related to an alteration in the basal degradative autophagy.

Surprisingly, p62 positive structures were not altered in the KO neurons, and they accumulated when treated with NMDA + CQ, showing that the absence of Ndr2 does not necessarily impair the bulk degradative autophagy. To provide an insight into other roles that autophagy has in the neurons, in which Ndr2 might be relevant, I analyzed the distribution of the APs within the neuron because it is well accepted that the majority of the degradative autophagosomes are formed in the axon tip and then transported retrogradely to soma to fuse with the late endosomes or the lysosomes to carry on degradation, the formation of autophagosomes is seldomly observed forming in the dendrites or the soma[23]; nonetheless, it has also been demonstrated that the formation of autophagosomes in the

spines is required for long-term depression in synaptic pruning and plasticity[66] and that the autophagosomes can also form in the spines and then transported retrogradely, showing that the localization of the autophagosomes within the neuron is subjected to a fine-tuned regulation that is dependent on the cellular context. We observed that the distribution of APs was altered in the Ndr2 KO neurons; more specifically, I saw an increase in LC3 positive structures in the secondary dendrites at the expense of a reduction in the primary ones. Additionally, when autophagy was induced with NMDA, the changes in the distribution in the dendrites were rescued, but the LC3 puncta in the soma increased when compared to the WT neurons. This could mean that under basal conditions, Ndr2 KO neurons have increased autophagosome formation in the dendrites and that upon stimulation of the recycling of the AMPA receptors with NMDA: these APs travel to the soma for degradation, where they accumulate, given that once the APs entre the soma, they cannot re-enter the dendrites or the axon. The observation that there is increased AP formation in the dendrites has big implications for plasticity, given that the majority of the synapses form connections within the dendrites. Supporting our previous results, I observed that the Ndr2 KO neurons had increased APs in the synapses, which is independent of the induction or blockage of autophagy when compared with the WT neurons. Finally, given that one of the downstream phosphorylation targets of Ndr2 is the Integrin β1 subunit and that it is required for AMPA receptor synaptic transmission, I tested the possibility that the integrin signalling could be impaired in the spines of the Ndr2 KO neurons. We observed that the Ndr2 KO neurons have decreased Integrin β1 subunit staining in the spines of both primary and secondary dendrites and that in the primary dendrites, it is unresponsive to the induction of autophagy, in contrast to the WT neurons. This suggests that autophagy might regulate the recycling of the Integrin β1 subunit from the dendritic spines and that the recycling is impaired in the absence of NDR2. This suggestion correlates with the fact that NDR2 is found in association with the integrin β1 subunit both in early and late endosomes[106], showing that Ndr2 is involved both in the phosphorylation of the integrin β1 subunit and the subsequent recycling through independent but probably intertwined mechanisms. Also, since it has been shown that Ndr2 and the Integrin β1 subunit are localized together in Rab5, and Rab11 vesicles, which are mainly endosomes, the lack of Ndr2 prevents the shuttling of the Integrin β1 subunit to the endosomes, so when NMDA stimulates endocytosis of the AMPAr, the Integrin β1 subunit fails to enter the endosomes for further degradation. Confirming that the hippocampal-related increase of APs translates to *in vivo*, I observed an increase in LC3 staining in the dorsal hippocampus that was even across the DG, CA1 and CA3 but not in the formation of puncta. We confirmed this by western blot from whole DG lysates, where I observed an increase in the total LC3 and an increasing trend in LC3 II and p62 protein levels. Finally, I also showed that Ndr2 KO mice had decreased staining of the phosphorylated Integrin β1 subunit in the DG and CA1. This suggests that while there is an evident increase in APs, the basal

autophagic flux is not necessarily altered in the hippocampus of adult mice but that the absence of Ndr2 partially impairs Integrin β1 subunit activation. In conclusion, Ndr2 deficiency increases the APs in the neurons' synapses and the LC3 protein levels in the DH of adult mice, while preventing the activation of the Integrin β1 subunit.

Previous works from our lab demonstrated that Ndr2 deficiency slightly impairs spatial memory in mice, which is hippocampus-dependent, and that Ndr2 KO mice have impairments in LTP in the CA1. Even though the molecular mechanism by which this occurs has not been thoroughly described, work from our group has shown that the LTP deficit can be rescued by the administration of extracellular matrix adhesion peptides like the Arginine-Glycine-Aspartate (RGD) peptides that mimic the integrin binding activity upon activation. Showing that integrin activation downstream of Ndr2 is required. The results summarized previously provide evidence that autophagy might be involved in the observed phenotype, given that autophagy is required for memory formation and synaptic plasticity. With that in mind, I decided to further investigate the effect that the manipulation of autophagy in the Ndr2 KO mice had on spatial learning and memory.

CHAPTER 2

PHARMACOLOGICAL UPREGULATION OF AUTOPHAGY WITH TREHALOSE IN THE DORSAL HIPPOCAMPUS IMPAIRS SPATIAL NAVIGATION AND MEMORY DURING AGEING

To determine if autophagy upregulation in the Ndr2 KO mice would impact memory and learning, I first established a model in which autophagy could be upregulated in the brain by administering the disaccharide trehalose. Trehalose was chosen because it is able to reduce protein aggregation in neurons in different models of neurodegenerative diseases such as AD and PD. Nonetheless, though there is compelling evidence that trehalose exercises its beneficial effects through the activation of autophagy, there is still conflicting evidence about its mechanism of action. It has been suggested that trehalose binds to the SL2CA family of proteins, which are glucose transporters, blocking them and thus creating a starvation state in the cells that leads to the increase of AMP and the activation of AMPK. Another proposed mechanism of action is the activation of the transcription factor EB (TFEB), a master regulator of the transcription of lysosomal genes, through an increase in lysosomal stress, TFEB dephosphorylation by the protein phosphatase 3, and its subsequent nuclear translocation. Nonetheless, to which extent trehalose can be used as an intervention to prevent the loss of spatial memory during ageing remains to be tested, but given that trehalose is easily synthesized from plants and it is already commercially available and widely used as a food supplement and sweetener, it has a high potential translatability value as a naturally occurring anti-ageing drug, and its effects on autophagy regulation *in vivo* should be further explored.

Chronic trehalose treatment increases autophagy in the dorsal hippocampus of old mice

In order to test the effect of trehalose in autophagy in the brain, young (3 months old) and old (18 months old and 24 months old), C57BL/6J mice were given trehalose 2.2% in the drinking water *ad libitum* for a period of 10 weeks, after which, the brains were extracted for protein biochemistry. Western blot was performed in dorsal hippocampus, ventral hippocampus and frontal cortex samples (Figure 11), given that these 3 brain regions are highly relevant in learning and memory, particularly for spatial and episodic memory. We observed that the trehalose treatment did not affect the protein expression of our targets in the young mice's brains; nonetheless, it increased TFEB protein levels in the DH of the old mice and in the FC of only the 24-month-old mice. Regarding total LC3 and LC3-II protein levels, I observed an opposite effect in the DH of the 18 and 24-month-old mice. Whereas protein levels were increased in the former, a significant decrease was observed in the latter. This was accompanied by a decrease of p62 protein levels in the 24-month-old mice, without changes in the 18-month-old

mice. These results altogether show that trehalose treatment increased autophagic flux, specifically in the DH of the 24-month-old mice, partially modulates autophagy in the 18-month-old group, and has no effect on autophagy in the brain of young mice.

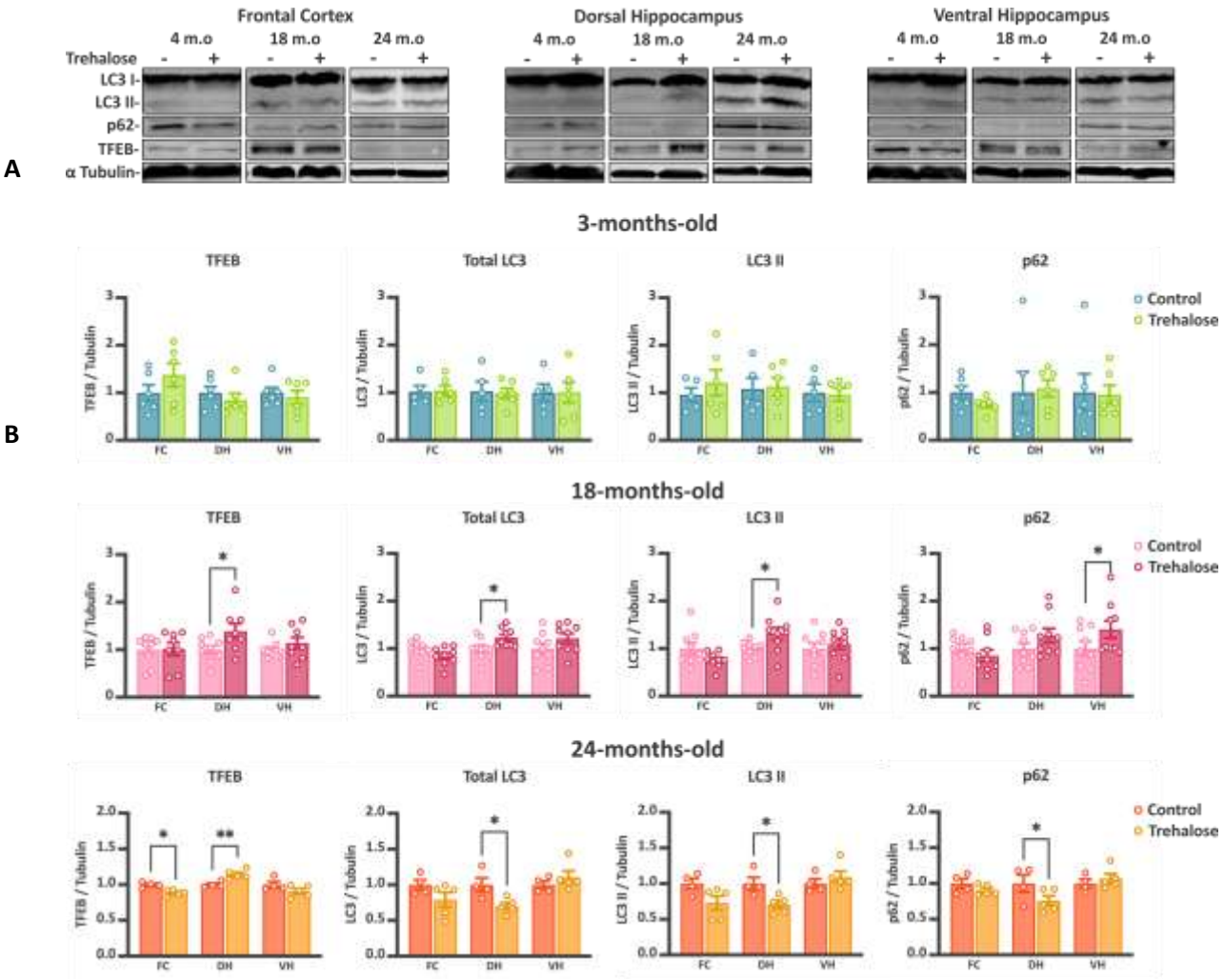


Figure 11. Chronic trehalose administration increases autophagic flux in the dorsal hippocampus of old mice. Young (3-months-old), old (18-months old and 24-months-old) mice were given water *ad libitum* supplemented with trehalose 2.2 % for a period of 10 weeks n=6 & 6; 9 & 8; 4 & 6, respectively. Brains were dissected, and western bot was performed in frontal cortex (FC), dorsal hippocampus (DH) and ventral hippocampus (VH) samples against LC3, p62 and TFEB (A). Total LC3, LC3 II, p62 and TFEB protein levels were quantified (B). The bars represent the mean ± S.E.M. Each dot represents the sample of each mice. *Two-way RM-ANOVA* with Bonferroni post-hoc comparison; *p<0.05, **p<0.005.

Lastly, to provide some insights into whether the observed increase in TFEB correlates with an increase in its transcriptional activity, another downstream target of TFEB was analyzed. It is known that TFEB is

a master transcription factor that is involved not only in the transcription of autophagy-related proteins but also in lysosomal and endosomal biogenesis through the regulation of the CLEAR (Coordinated Lysosomal Expression and Regulation) network of genes, which includes more than 470 targets like *Becn1* and *Lamp1* and has been suggested to be a primary effector of autophagy induction upon trehalose treatment. To investigate whether this might be the mechanism of action of chronic trehalose administration in the DH, LAMP1 protein levels were analyzed by WB. LAMP1 protein levels remained unchanged in the DH of young mice, and though not significant, a trend to increase was observed in the old mice (Figure 12), which suggests that the effect of autophagy induction by trehalose in the DH of old mice, may be carried out by an increase in TFEB´s transcriptional activity.

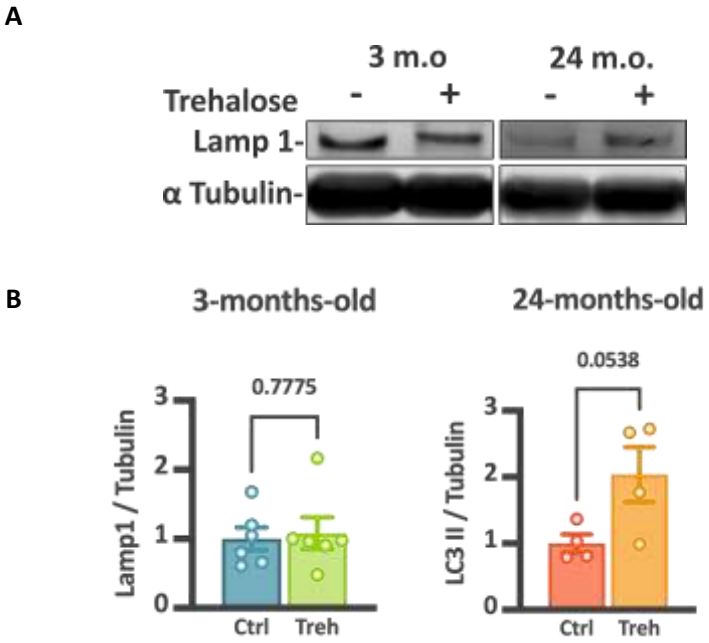
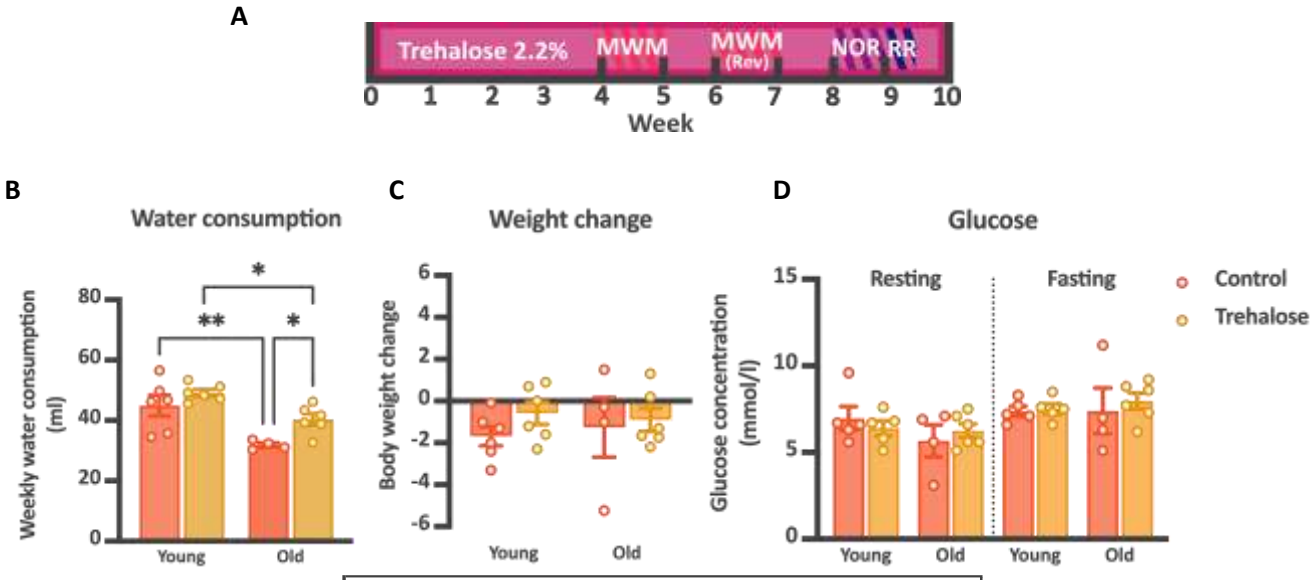


Figure 12. Chronic trehalose administration increases LAMP1 protein levels in the hippocampus of old mice. Young (3-months-old) and old (24-months-old) mice were given water *ad libitum* supplemented with trehalose 2.2 % for a period of 10 weeks. Brains were dissected, and western bot was performed in dorsal hippocampus samples against LAMP1 (A) and protein levels were quantified (B). The bars represent the mean ± S.E.M. Each dot represents the sample of each mice. *Two-tailed unpaired Student´s t-test,* *p<0.05. n=6 & 6 (young) 4 & 6 (old).

Overall, this shows that chronic trehalose treatment can increase the basal autophagic flux in the DH in an age-dependent manner, possibly through the upregulation of TFEB. Still, in order to evaluate the effect that the pharmacological upregulation of autophagy has on cognition, mice were subjected to a behavioural paradigm to test hippocampal-dependent memory. Young and old mice were given trehalose for a period of 10 weeks, during which they were subjected to training in the Morris water

maze (MWM) plus reversal learning, spontaneous alternation, (Sp. Alt.) novel object recognition in the Y maze (NOR) and rotarod (RO), the weight and water consumption were monitored weekly. Even though trehalase, the enzyme that cleaves trehalose into 2 glucose molecules, is only present in small quantities in the intestine, I also monitored the possibility that trehalose treatment could increase blood sugar, so on the last day of treatment, glucose levels were measured in basal conditions and after 8 hours of fasting (Figure 13). The young, both control and trehalose-treated mice, consumed the same amount of water every week, but the old mice tended to consume less water than the young ones regardless of the treatment; nonetheless, the old mice that were given trehalose consumed more than the old mice that were given plain water, probably due to the fact that trehalose has a sweet flavour. Despite that fact, trehalose consumption had no effect on the resting or fasting glucose levels between groups. We only observed a general increase in the glucose levels after fasting that was the same regardless of age and treatment, showing also that old mice had no evident alterations in metabolism. We also observed that during the 10 weeks of treatment, on average, mice gradually lost weight, although no differences between age and treatment were observed. This relates to the fact that mice were single-caged at the beginning of the treatment, which has been demonstrated to reduce body mass[142] and that mice underwent demanding physical activity through the 6 weeks of training.



E

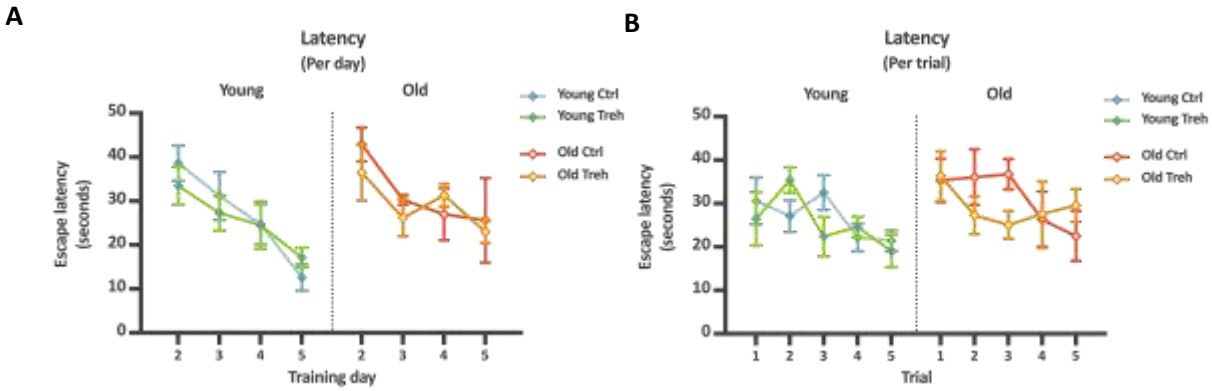
Glucose levels			
Source of Variation	Variation	P value	
Age	0,695%	0,5002	ns
Time	19,030%	0,0264	*
Treatment	0,381%	0,6167	ns
Age x Time	2,855%	0,3575	ns
Age x Treatment	1,934%	0,2668	ns
Time x Treatment	0,349%	0,7448	ns
Age x Time x Treatment	0,366%	0,7388	ns

Figure 13. Chronic trehalose treatment doesn't produce changes in weight or glucose levels. Young (3-months-old) and old (24-months-old) mice were given water *ad libitum* supplemented with trehalose 2.2 % for a period of 10 weeks, during which they were subjected to training in the Morris water maze (MWM) and reversal learning, spontaneous alternation, novel object recognition (NOR) and rotarod (RR) (A) Water consumption (B) and weight (C) were monitored weekly. *Two-way RM-ANOVA* with Bonferroni post-hoc comparison; *p<0.05, **p<0.005. One day before the end of treatment, glucose was measured in resting conditions and after 8 hours of fasting (D). The bars represent the mean ± S.E.M. Each dot represents each mouse. *Three-way RM-ANOVA* with Tukey post-hoc comparison (E). n=6 & 6 (young) 4 & 6 (old).

Chronic trehalose treatment impairs spatial memory in old mice.

The first paradigm that was conducted was the MWM, a task designed to evaluate spatial learning on the first days of the experiment (training) and spatial memory on the last day (probe trial). During the training, I observed that the treatment had no effect on the time needed to reach the platform (latency) in spite of the age and that the latency to reach the platform went down after each training day, showing that old and young mice learnt the task equally. Each training day consisted of 5 trials, so another way

to analyze short-term memory in the MWM is to compare the average latency of the same trial of each day. We didn´t observe any differences between groups, only a reduction in the latency after each consecutive trial indicating that trehalose treatment had no effects on short-term memory (Figure 14). One day after training, during the probe trial, the platform was removed, and the mice were left to swim freely for the duration of the test. The time spent in the target quadrant where the platform was located, the southwest (SW) quadrant, was quantified (Figure 15). Here I found that the trehalose treatment had no effects on the permanence in the young mice but that it decreased the permanence in the old mice when compared to young mice treated with trehalose or old mice that were given plain water. One week after the acquisition in the MWM, mice underwent reversal learning, where the platform was changed from the SW quadrant to the northeast (NE) quadrant, which was the corner most opposite (Figure 16). During training, and consistent with what I found in the acquisition, nonetheless, the old mice took more time overall to reach the platform regardless of treatment when the latency was analyzed by each training day. Also consistent with what I observed in the acquisition, neither age nor trehalose affected the latency to reach the platform by trial. Finally, during the probe trial, the trehalose treatment decreased the permanence in the escape quadrant (NE) of the old mice when compared to the young ones (Figure 17).



C

Latency per day			
Source of Variation	Variation	P value	
Day	27,87%	<0,0001	****
Age	2,85%	0,151	ns
Treatment	0,47%	0,551	ns
Day x Age	2,23%	0,4017	ns
Day x Treatment	1,76%	0,5061	ns
Age x Treatment	0,04%	0,8568	ns
Day x Age x Treatment	0,72%	0,8108	ns
Mice	22,80%		

Latency per trial			
Source of Variation	Variation	P value	
Day	9,64%	0,0123	*
Age	3,26%	0,1588	ns
Treatment	0,56%	0,5489	ns
Day x Age	1,05%	0,8256	ns
Day x Treatment	4,50%	0,1806	ns
Age x Treatment	0,05%	0,8535	ns
Day x Age x Treatment	3,93%	0,2398	ns
Mice	27,15%		

Figure 14. Chronic trehalose treatment doesn't produce changes in the escape latency of the MWM during acquisition. Young (3-months-old) and old (24-months-old) mice were subjected to training in the Morris water maze. The latency to reach the platform on each day (A) and each trial (B) was analyzed. The dots represent the mean ± S.E.M. Each dot represents the average of all respective trials of each mouse. *Three-way RM-ANOVA* with Tukey post-hoc comparison (C). n=6 & 6 (young) 4 & 6 (old).

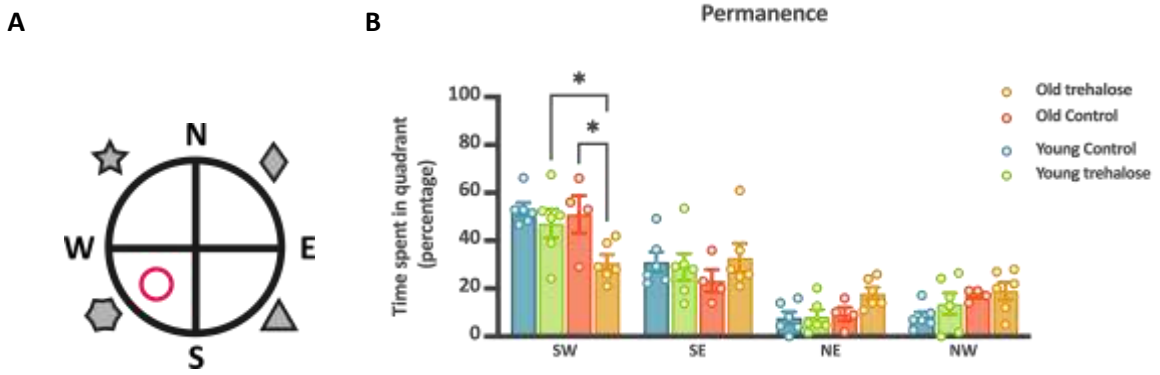
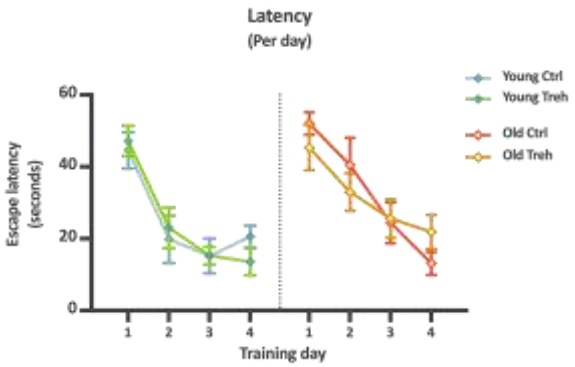
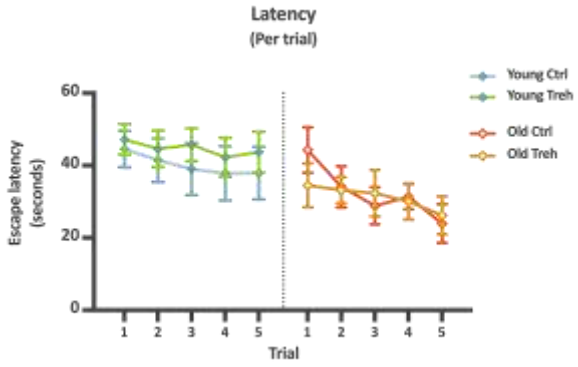


Figure 15. Chronic trehalose treatment decreases permanence in the escape quadrant of the MWM during ageing. Young (3-months-old) and old (24-months-old) mice were subjected to training in the Morris water maze. In the probe trial, the platform was removed from its location in the SW quadrant (A) and the time spent in each of the quadrants was quantified (B). The bars represent the mean ± S.E.M. Each dot represents each mouse. *Two-way RM-ANOVA with* Tukey post-hoc comparison; *p<0.05, **p<0.005. n=6 & 6 (young) 4 & 6 (old).

A



B



C

Latency per day			
Source of Variation	Variation	P value	
Day	48,87%	<0,0001	****
Age	4,46%	0,0915	ns
Treatment	0,04%	0,8665	ns
Day x Age	3,06%	0,0243	*
Day x Treatment	0,18%	0,8962	ns
Age x Treatment	0,01%	0,9215	ns
Day x Age x Treatment	2,50%	0,0505	ns
Mice	25,25%		

Latency per trial			
Source of Variation	Variation	P value	
Day	5,29%	<0,0001	****
Age	13,93%	0,0651	ns
Treatment	0,34%	0,7629	ns
Day x Age	1,20%	0,1415	ns
Day x Treatment	1,13%	0,1626	ns
Age x Treatment	1,05%	0,5956	ns
Day x Age x Treatment	0,36%	0,7049	ns
Mice	64,98%		

Figure 16. Chronic trehalose treatment doesn't produce changes in the escape latency of the MWM during reversal learning. Young (3-months-old) and old (24-months-old) mice were subjected to training in the Morris water maze. The latency to reach the platform on each day (A) and each trial (B) was analyzed. The dots represent the mean ± S.E.M. Each dot represents the average of all respective trials of each mouse. Three-way RM-ANOVA with Tukey post-hoc comparison (C). n=6 & 6 (young) 4 & 6 (old).

A

B

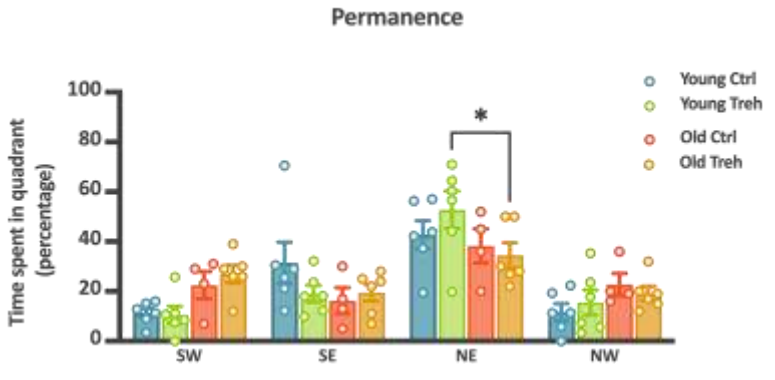


Figure 17. Chronic trehalose treatment decreases permanence in the escape quadrant of the MWM during reversal learning in ageing. Young (3-months-old) and old (24-months-old) mice were subjected to training in the Morris water maze. In the probe trial, the platform was removed from its location in the NE quadrant (A) and the time spent in each of the quadrants was quantified (B). The bars represent the mean ± S.E.M. Each dot represents each mouse. *Two-way RM-ANOVA with* Tukey post-hoc comparison; *p<0.05, **p<0.005. n=6 & 6 (young) 4 & 6 (old).

One week after reversal learning, mice were placed in a Y-maze and left to explore freely for 8 minutes (Figure 18). The spontaneous alternation test is used to evaluate spatial working memory, and it relies on the ability of the mice to remember which of the 3 arms in the Y-maze they have visited less in a given sequence. The percentage of alternations, which means a correct entry sequence from the least visited arm to the most visited, e.g. Arm A → Arm C → Arm B→ was calculated as well as the number

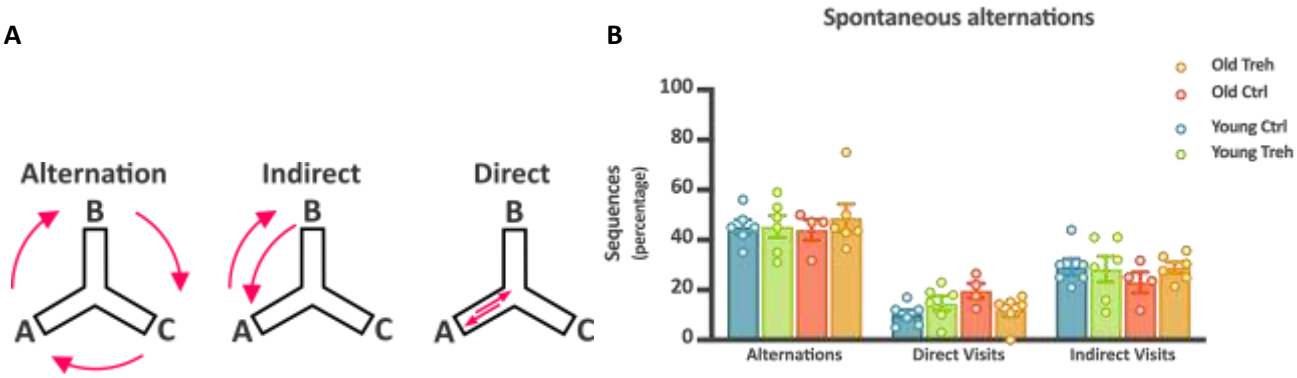


Figure 18. Chronic trehalose treatment doesn´t produce changes in spontaneous alternation. Young (3-months-old) and old (24-months-old) mice were left to explore a Y-maze freely for 8 minutes. The entries to each arm were recorded, and the number of alternations, indirect visits and direct visits (A) were counted, and the percentage of each kind of sequence was calculated (B). The bars represent the mean ± S.E.M. Each dot represents each mouse. *Two-way ANOVA*. n=6 & 6 (young) 4 & 6 (old).

of direct entries (entering the same arm twice), and indirect entries (entering the second most visited arm). One day after the spontaneous alternation in the Y-maze, mice were subjected to the novel object recognition (NOR) paradigm in the Y-maze (Figure 19). During the NOR task, the mice were habituated to 2 similar objects for a period of 8 minutes. Then, one of the objects was removed and replaced with a new one, and the mice were placed back in the Y-maze to explore the objects for 8 more minutes. Naturally, if the mice remember the old object, they will favour the exploration of the new one; in the same way, if the mice spend the same amount of time exploring the new and the old object, it is considered that the mice have impairments in spatial memory and are not able to discriminate new landmarks in the maze. The Discrimination Index (D.I.) is a measure that represents the ability of each individual mouse to distinguish between two objects with a numerical value between 1 and -1. Hence, a positive value reflects a preference for the new object, a negative value for the old object, and a value close to 0, the inability to discriminate. We found that whereas young mice did not discriminate between the two objects, the trehalose treatment impaired the discrimination ability of the old ones.

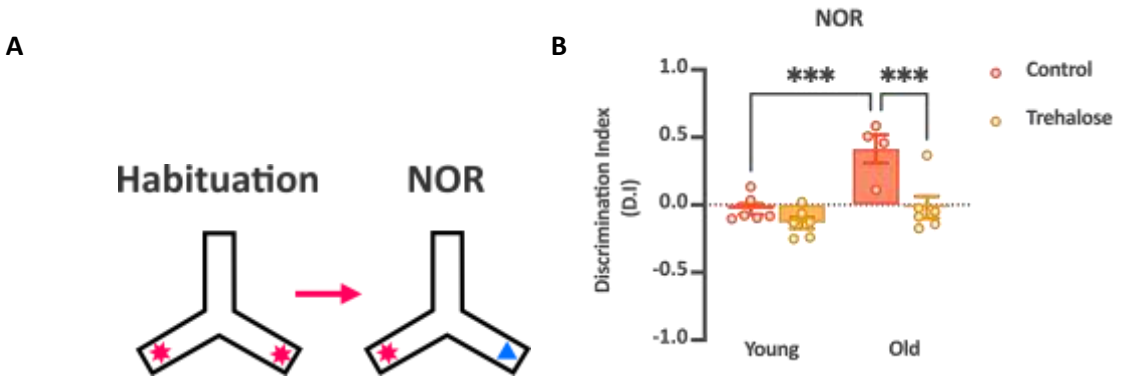
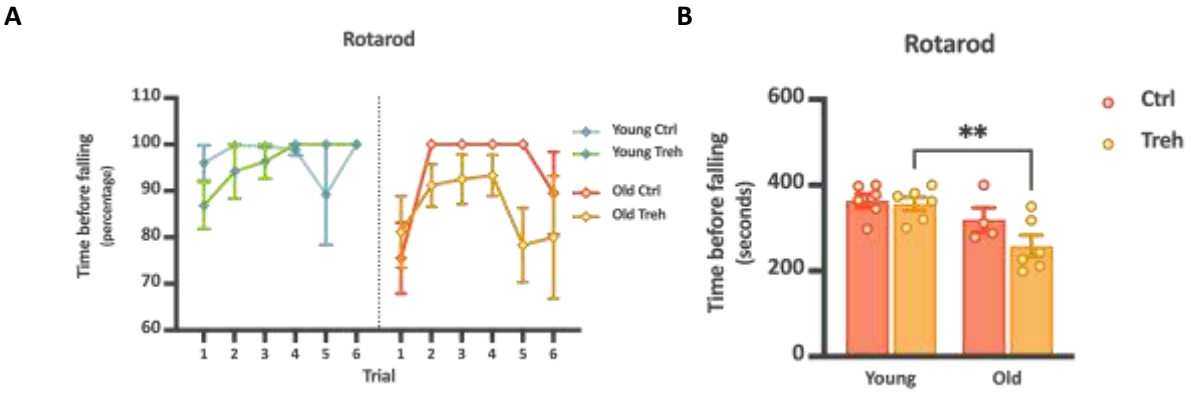


Figure 19. Chronic trehalose treatment impairs the discrimination between objects in the NOR during ageing. Young (3-months-old) and old (24-months-old) mice were habituated to 2 similar objects in a Y-maze for 8 minutes, after that, one object was replaced for a new one (A) The time spent exploring each of the objects was measured and the discrimination index was calculated (B). The bars represent the mean ± S.E.M. Each dot represents each mouse. *Two-way RM-ANOVA*, ***p<0.0005. n=6 & 6 (young) 4 & 6 (old).

Finally, to test whether motor function might be impaired and contributing to the alterations observed in the MWM, motor memory and learning, mice were subjected to training in the rotarod (Figure 20). Each day the time spent in the rotarod before falling was quantified; on the first day of training, the rotation remained at a constant 15 revolutions per minute (rpm), and for the rest of the days, the rotation increased from 15 to 35 rpm. On the final day, the mice were left for 400 seconds with an increasing speed of 4 to 40 rpm. Even though the young ones outperformed the old mice during training, the administration of trehalose had no effects on the motor learning capabilities of the mice; nonetheless, during the probe trial, the old mice that underwent treatment remained for less time in the rotarod than the young treated ones. This shows that trehalose had no effects on motor learning and only a mild effect on motor function when mice were forced to walk.

In summary, the pharmacological upregulation of autophagy with trehalose impaired spatial memory, as was demonstrated in the MWM plus the reversal and the NOR tasks. Contrary to what I expected and what has been published in the literature, autophagy has been shown to be required for learning, and particularly that increasing autophagy during ageing prevents the normal reduction in memory[12].



C

Latency per trial			
Source of Variation	Variation	P value	
Trial	9,65%	0,0091	**
Age	5,28%	0,0356	*
Treatment	2,51%	0,1348	ns
Trial x Age	3,70%	0,2901	ns
Trial x Treatment	0,40%	0,9835	ns
Age x Treatment	1,50%	0,241	ns
Trial x Age x Treatment	5,82%	0,0893	ns
Mice	18,41%		

Figure 20. Chronic trehalose treatment mildly impairs performance in the rotarod during ageing. Young (3-months-old) and old (24-months-old) mice were trained in the rotarod. The percentage of time spent in the rotor before falling during the training phase was calculated (A) *Three-way RM-ANOVA* with Tukey post-hoc comparison (B), then during the probe trial, the time was increased to 400 seconds, and the time before falling was measured (C). The bars represent the mean ± S.E.M. Each dot represents each mouse. *Two-way RM-ANOVA*, ***p<0.0005. n=6 & 6 (young) 4 & 6 (old).

Autophagy has also been shown to be upregulated during learning, and transgenic mice that have impairments in autophagy, display worse memory performance. With that taken into account, I pondered the possibility that alterations in the navigation strategies used to find the platform in the MWM could be a contributing factor to the observed decrease in permanence and not only impairment in spatial memory.

Chronic treahlose treatment changes spatial navigation strategies in old mice

It has been demonstrated that when rodents are trained to find the platform in the MWM, as the training progresses, and they learn the spatial location of the platform, they use different navigation strategies to reach it. In the first stages of learning, when the mice have not been acquainted with the platform, they will swim in circles close to the rim of the maze, trying to find a way out of the adverse stimulus, which is the water. In the following sessions, once the mice learn that there is an escape to the maze, but they have not acquired the spatial memory of the location of the platform, they will try to find it using a combination of mainly 3 different kinds of egocentric (non-dependent on the cues) strategies; random search, scanning, and chaining. Scanning is when the mice swim in a wide trajectory from the starting position to one end of the maze, then back close to the starting position, and to the

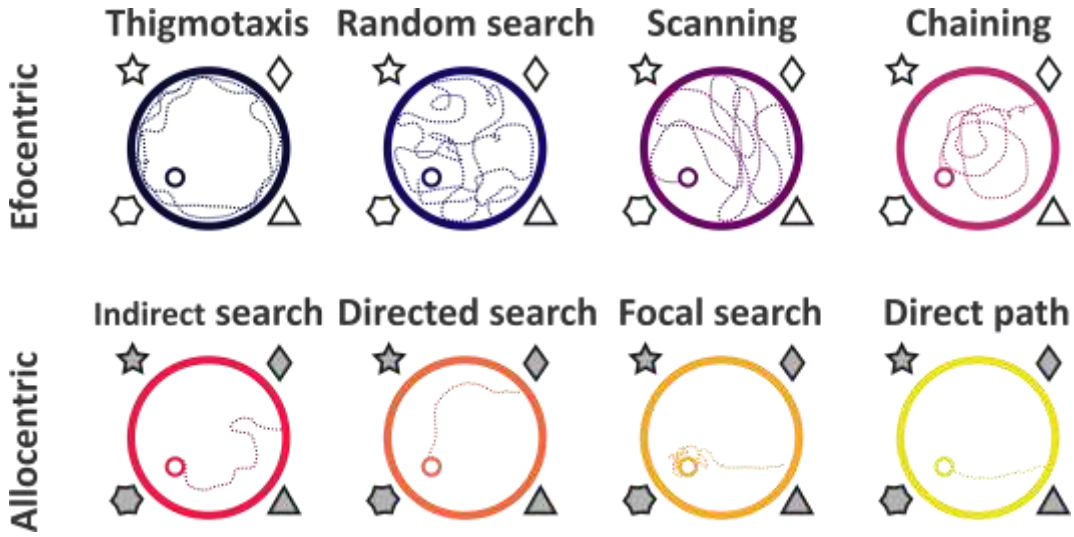
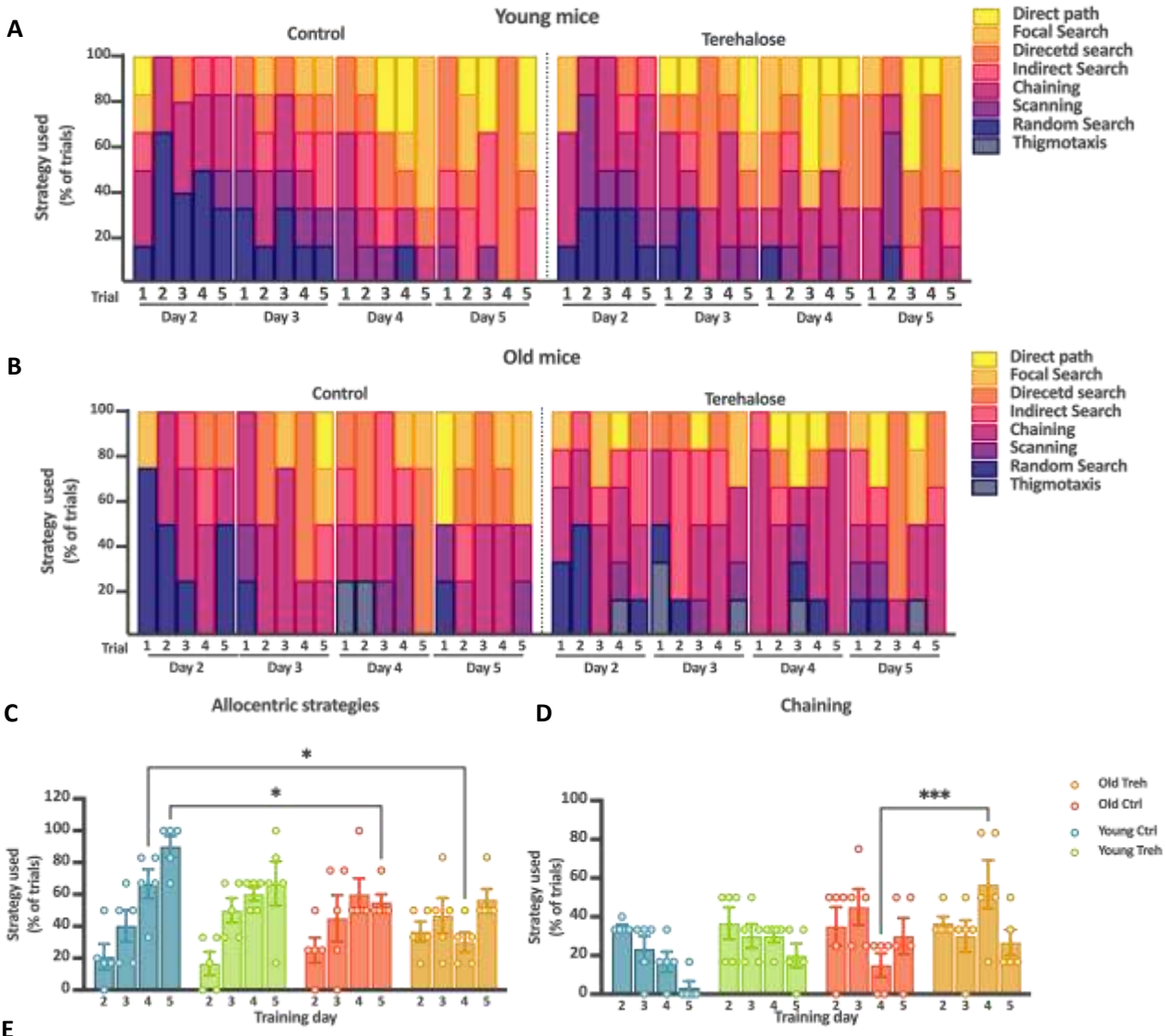


Figure 21. Navigation strategies used in the Morris Water Maze. The navigation strategies were analyzed using the Python script pathfinder, and were defined as following; Thigmotaxis: 50% of the time spent in the thigmotaxis area (15 cm away from the rim), random search: Minimum 50% of the maze traversed, scanning: Minimum 25% of the maze traversed and maximum 75% with an average distance of 56,5 cm from the center of the maze, chaining: Maximum 80% of the maze traversed and at least 75% of the time spent in the annulus zone (15 cm away from the thigmotaxis area, and 20 cm away from the center of the maze), indirect search: Maximum 90° of heading error, at an ideal path error (IPE) of 75 cm, directed search: Maximum travel distance of 900 cm and 70% of the time spent facing the platform center in a 40° angle, focal search: Maximum travel distance of 450 cm, at an average distance of 36,5 cm from the platform center and direct path: IPE of 150 cm with a heading error of maximum 30°.

rim of the maze again, traversing a broad proportion of the maze until the platform is found. On the other hand, chaining refers to a strategy that involves swimming in concentric circles that can widen or get narrower until the mice collide with the platform. The next stage of learning involves the acquisition of allocentric strategies, which is when a mental map of the maze and the platform has formed with respect to the external visual cues. In this case, they are divided into 4 main categories that take into consideration the search distance from the platform and the efficiency of the path taken to reach it. In order of less to more efficient with less to more area searched, they are the following: Indirect search, focal search, directed search and direct path (Figure 21). The navigation strategies used in each trial during the acquisition in the MWM were analyzed in this fashion using the Python script "Pathfinder" (Figure 22). I observed that all the mice increased the usage of allocentric strategies as the training went on; nonetheless, in the old mice, the increase peaked at the 4th day of training and even more, the trehalose treatment decreased the usage of allocentric strategies in the old mice. This went on hand with a gradual reduction of chaining in the young mice and a preference for chaining in the old mice, which was accentuated by the trehalose treatment. Accordingly, when I analyzed the probe trial by dividing it into cumulative time bins (first 10, 20, 30 and 60 seconds), I saw that the trehalose treatment increased the percentage of chaining in the first 20 seconds of the probe trial only in the old mice (Figure 23). Seeing that the trehalose had an effect on the exploratory behaviour, the strategies used during the reversal learning of the MWM were analyzed in the same fashion (Figure 24). In the same way as what I observed during the acquisition, the percentage of allocentric strategies used increased on each training day. What was different from the acquisition was that the young mice treated with trehalose had higher usage of allocentric strategies on the last day of training compared to the untreated mice, and t among all the groups, the old mice treated with trehalose displayed the least allocentric navigation preference. These changes were also accompanied by an increase in chaining, consistent with what occurred during the acquisition. But surprisingly, when the probe trial was analyzed, no differences were observed between age or treatment. These results altogether indicate that the changes that trehalose treatment produced in the exploratory behaviour contributed to the decreased permanence during the acquisition in the MWM but do not explain the decreased permanence during the reversal learning.

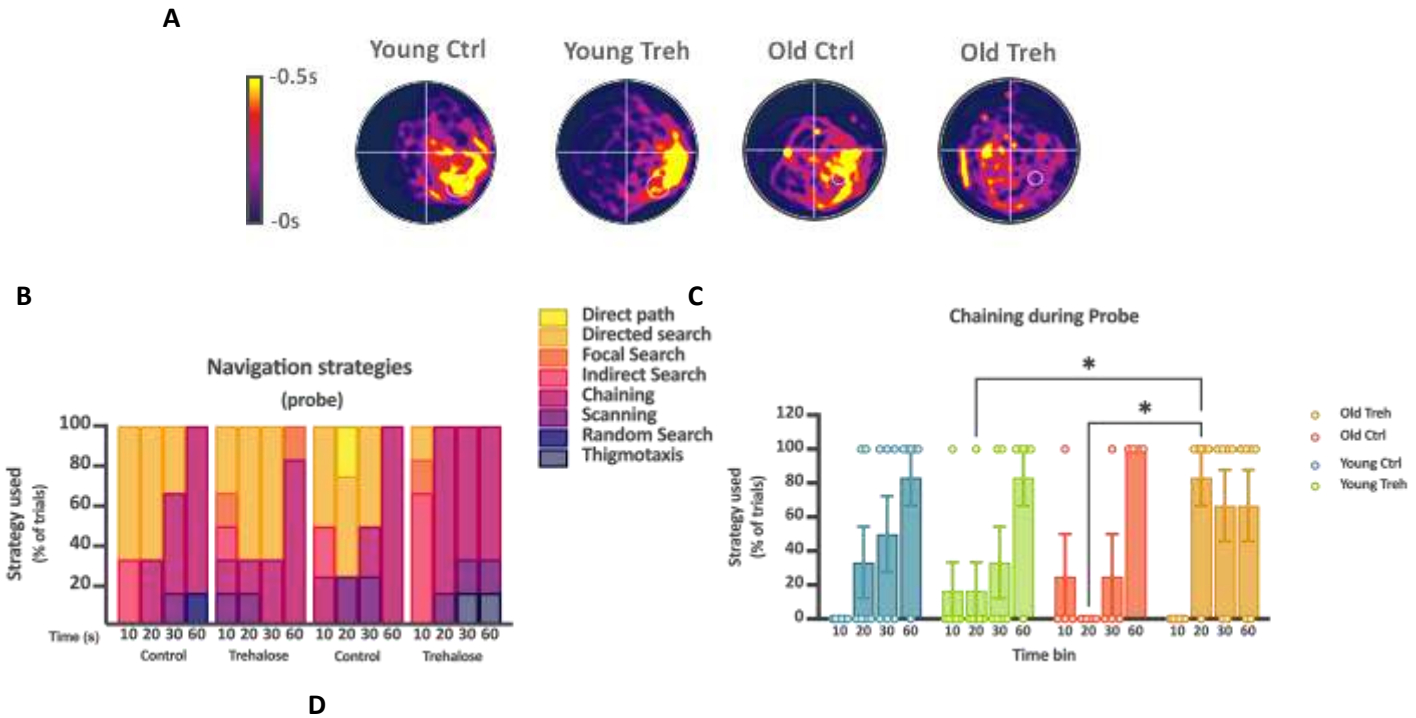


E

Allocentric strategies			
Source of Variation	Variation	P value	
Day	35,53%	<0,0001	****
Age	1,85%	0,178	ns
Treatment	0,89%	0,3427	ns
Day x Age	7,46%	0,0176	*
Day x Treatment	3,73%	0,1499	ns
Age x Treatment	0,05%	0,8248	ns
Day x Age x Treatment	3,44%	0,177	ns
Mice	14,89%		

Chaining			
Source of Variation	Variation	P value	
Time	27,49%	<0,0001	****
Age	0,39%	0,6528	ns
Treatment	0,39%	0,6528	ns
Time x Age	0,39%	0,8387	ns
Time x Treatment	3,47%	0,0667	ns
Age x Treatment	1,07%	0,4556	ns
Time x Age x Treatment	8,96%	0,0008	***
Mice	33,19%		

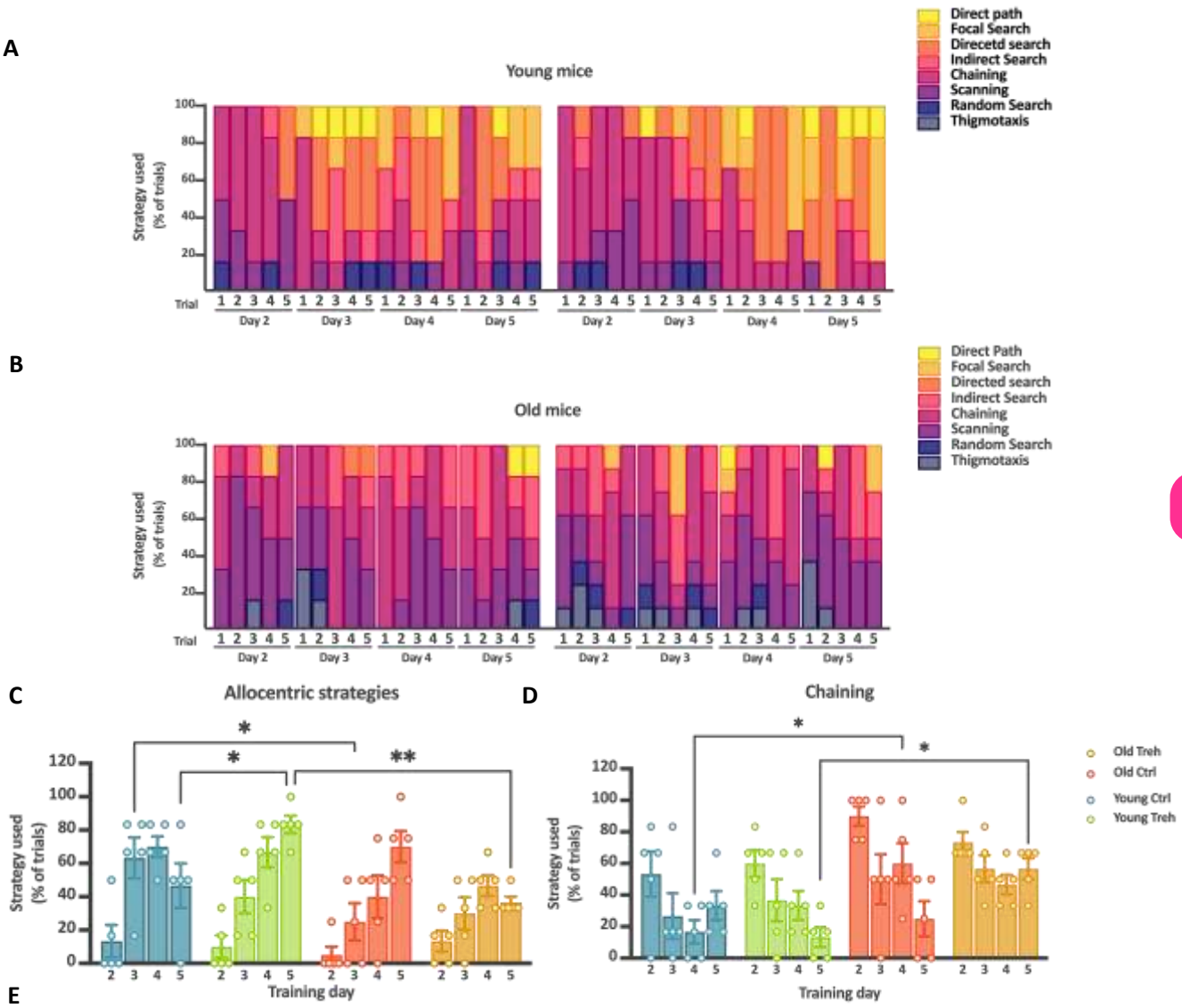
Figure 22. Chronic trehalose treatment prevents the acquisition of allocentric navigation strategies, and increases chaining during ageing. Young (3-months-old) and old (24-months-old) mice were trained in the MWM. The navigation strategies used during the learning phase of the acquisition were divided into direct path, focal search, directed search, indirect search, chaining, scanning, random search and thigmotaxis (A). The allocentric strategies (direct path, focal search, directed search and indirect search) were evaluated by each training day (B), as well as the chaining. The bars represent the mean ± S.E.M. Each dot represents each trial. *Two-way RM-ANOVA* with Tukey post-hoc comparison; *p<0.05, ***p<0.0005 (C). *Three-way RM-ANOVA* with Tukey post-hoc comparison (D). n=6 & 6 (young) 4 & 6 (old).



D

Chaining			
Source of Variation	Variation	P value	
Time	27,49%	<0,0001	****
Age	0,39%	0,6528	ns
Treatment	0,39%	0,6528	ns
Time x Age	0,39%	0,8387	ns
Time x Treatment	3,47%	0,0667	ns
Age x Treatment	1,07%	0,4556	ns
Time x Age x Treatment	8,96%	0,0008	***
Mice	33,19%		

Figure 23. Chronic trehalose treatment reduces the use of allocentric navigation in the probe trial of the MWM, and increases chaining during ageing. Young (3-months-old) and old (24-months-old) mice were trained in the MWM. The navigation strategies used during the probe trial (A) of the acquisition were divided into direct path, focal search, directed search, indirect search, chaining, scanning, random search and thigmotaxis and analyzed by cumulative time bins (0-10s, 0-20s, 0-30s and 0-60s) (B). The percentage of chaining used in each time bin was analyzed. The bars represent the mean ± S.E.M. Each dot represents each mouse. *Two-way RM-ANOVA* with Tukey post-hoc comparison, *p<0.05 (C). *Three-way RM-ANOVA* with Tukey post-hoc comparison (D). n=6 & 6 (young) 4 & 6 (old).

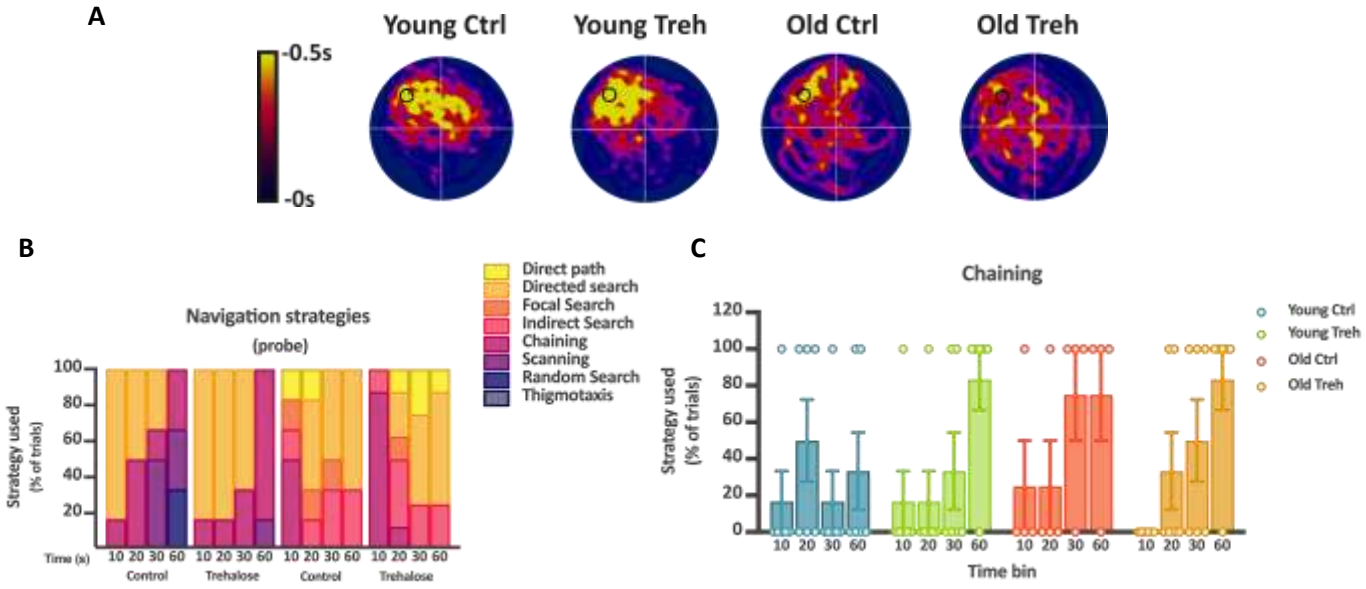


E

Allocentric strategies			
Source of Variation	Variation	P value	
Day	42,23%	<0,0001	****
Age	7,13%	0,0141	*
Treatment	0,02%	0,886	ns
Day x Age	2,48%	0,1585	ns
Day x Treatment	0,66%	0,6963	ns
Age x Treatment	0,18%	0,6689	ns
Day x Age x Treatment	10,37%	0,0003	***
Mice	15,01%		

Chaining			
Source of Variation	Variation	P value	
Day	23,67%	0,0001	***
Age	16,04%	0,0006	***
Treatment	0,22%	0,6266	ns
Day x Age	0,48%	0,8887	ns
Day x Treatment	0,76%	0,8017	ns
Age x Treatment	0,01%	0,9105	ns
Day x Age x Treatment	7,72%	0,0261	*
Mice	14,38%		

Figure 24. Chronic trehalose treatment reduces the acquisition of allocentric navigation strategies in the reversal learning of the MWM, and increases chaining during ageing. Young (3-months-old) and old (24-months-old) mice were trained in the MWM. The navigation strategies used during the learning phase of the reversal learning were divided into direct path, focal search, directed search, indirect search, chaining, scanning, random search and thigmotaxis (A). The allocentric strategies (direct path, focal search, directed search and indirect search) were evaluated by each training day (B), as well as the chaining. The bars represent the mean ± S.E.M. Each dot represents each trial. *Two-way RM-ANOVA* with Tukey post-hoc comparison, *p<0.05, **p<0.005 (C). *Three-way RM-ANOVA* with Tukey post-hoc comparison (D). n=6 & 6 (young) 4 & 6 (old).



D

Allocentric strategies			
Source of Variation	Variation	P value	
Time	15,98%	<0,0001	****
Age	1,60%	0,4351	ns
Treatment	0,00%	>0,9999	ns
Time x Age	3,20%	0,1047	ns
Time x Treatment	3,02%	0,1206	ns
Age x Treatment	0,71%	0,6011	ns
Time x Age x Treatment	3,02%	0,1206	ns
Mice	45,13%		

Figure 25. Chronic trehalose increases the preference for chaining in the reversal learning of the MWM during ageing. Young (3-months-old) and old (24-months-old) mice were trained in the MWM. The navigation strategies used during the probe trial (A) of the reversal were divided into direct path, focal search, directed search, indirect search, chaining, scanning, random search and thigmotaxis and analyzed by cumulative time bins (0-10s, 0-20s, 0-30s and 0-60s) (B). The percentage of chaining used in each time bin was analyzed. The bars represent the mean ± S.E.M. Each dot represents each mouse. *Two-way RM-ANOVA* with Tukey post-hoc comparison (C). *Three-way RM-ANOVA* with Tukey post-hoc comparison (D). n=6 & 6 (young) 4 & 6 (old).

Discussion

In this chapter, I set up a model of pharmacological upregulation of autophagy to evaluate the effects it had on learning and memory that could be applied to other mice models, such as the Ndr2 KO mice. I treated mice of 3 different age groups (3, 18 and 24 months old) with trehalose 2.2% in the drinking water, then I evaluated the basal autophagic flux in the DH, VH and FC. While Trehalose increased the autophagic flux in the DH hippocampus of the old (24-months-old) mice, it failed to induce any changes in other brain regions of the young mice (3-months-old) and only mildly altered basal autophagy in the DH of the 18 months old mice, the changes in autophagy correlated with a strong increase in TFEB protein levels. Given that the main effect occurred in the DH, I analyzed hippocampal-dependent memory by subjecting the mice to the MWM, Spontaneous alternation and NOR. I observed that trehalose treatment decreased the permanence in the escape quadrant in the MWM and one week after, in the reversal learning without changes in the learning curve only in the old mice. Moreover, trehalose also decreased the discrimination index of the old mice during the NOR in the Y-maze without producing changes in the spontaneous alternation task. Finally, I analyzed the exploratory behaviour by classifying the paths used to navigate the MWM during learning and in the probe trial. Here I demonstrated that trehalose increased the chaining preference during the acquisition, probe trial and reversal learning in the old mice at the expense of the use of allocentric strategies. I can conclude that the trehalose treatment increases autophagy in the DH of old mice and impairs spatial memory by producing changes in exploratory behaviour.

The age-dependent effect on autophagy upregulation by trehalose could be due to the fact that as I age, autophagy becomes dysregulated, meaning that the mechanisms that coordinate autophagy in its many different control points such as the Beclin 1 complex, ULK1 complex, the ATG5/12 conjugation system, Fusion with the lysosome and degradation, and all the pathways that converge on them, become altered unevenly. The regulation of autophagy is a very complex system; thus, signalling-wide alterations are harder to predict. The functional implication of these alterations has been highly discussed because the outcome can include what seems at a glance as contradicting observations like; protein accumulation and increased protein degradation, metabolic dysfunction and decreased signalling, loss of stemness and malignant cellular transformation, etc. All given, it is clear that what is

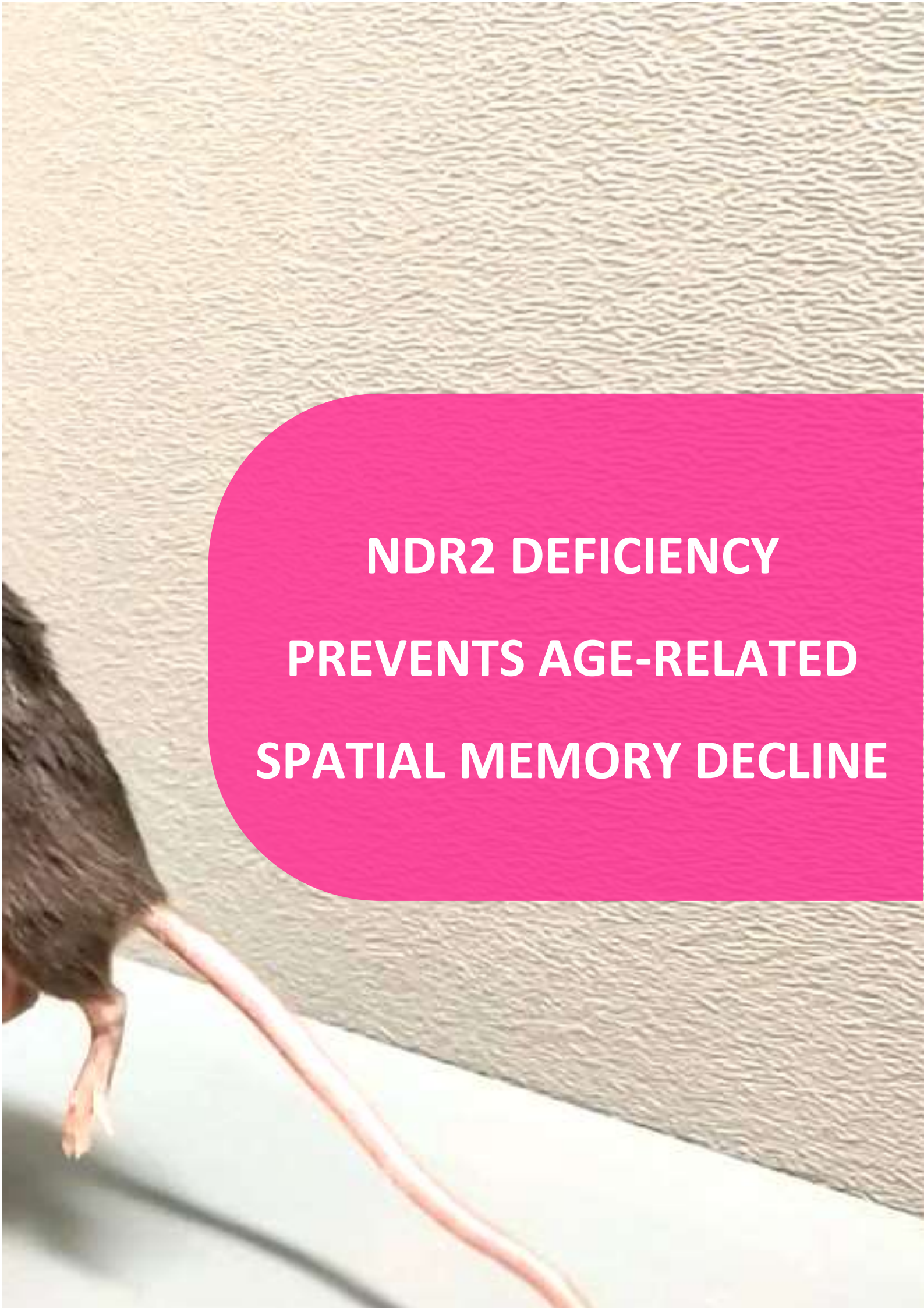
lost during ageing is the robustness in the regulation of autophagy. For this reason, it is expected that a mild pharmacological stimulus, such as trehalose, contrary to a strong one like genetic manipulation or drugs administered *in situ,* such as what has been reported before with the tat-Beclin 1 peptide, failed to induce autophagy in the brains of the young mice, but had a strong effect in the oldest ones, where the robustness in autophagy regulation has been already lost, and a mild and inconclusive effect in the 18-months-old mice, where some mechanisms might be altered, and some others not completely. Regarding trehalose, even though the mechanism of action by which trehalose increases TFEB activity is still under investigation, it is commonly accepted that in order to transcribe the genes from the CLEAR network, the cytoplasmic TFEB has to be de-phosphorylated and translocated to the nucleus. Some of the most frequent stimuli that produce this translocation are amino acid starvation, inhibition of mTOR with rapamycin and endoplasmic reticulum or lysosomal stress[143]. Nonetheless, it has been demonstrated that TFEB can also be regulated by protein stabilization; Phosphorylation of TFEB at the S462/463/467/469 by PKCB targets it to proteasomal degradation through the E3 ligase Hsc70/STUB1[144], and in conditions such as starvation. Given that neurons are energetically highly demanding, they have evolved alternative mechanisms to cope with nutrient deprivation that rely on the usage of ketone bodies and highly efficient ketogenesis controlled by AMPK[145], and it is known that under starvation conditions, they die easily. So, here I also provide evidence that besides the nuclear translocation, trehalose could also promote the stabilization of TFEB to ease its translocation and subsequent transcription of its downstream targets. Nonetheless, this would have to be investigated further.

When I analyzed the spatial memory in the MWM, I observed that the learning and the short-term memory were not affected by trehalose, and even more, that young and old mice learnt in a comparable way. But in a different outcome to what was expected, considering that autophagy has been shown to be beneficial for memory formation, I observed that the upregulation of autophagy by trehalose affected the permanence in the escape quadrant both in the acquisition and the reversal probe. One possibility that I analyzed was that the decreased permanence could be due to the fact not that the mice didn´t remember where the platform was but that the mice apparently spent less time looking for the platform close to where it was, but because they were looking for it in a different way, in accordance to that, trehalose increased the use of chaining and decreased the use of allocentric strategies. The idea that the way in which old people navigate changes with ageing has been investigated before. Although some studies seem contradictory upon which kind of navigation (egocentric vs allocentric) is a predictor

of better memory performance during ageing, what seems to hold up is that in the process of creating a visual representation of a location in a context, the use of egocentric strategies precedes the acquisition of allocentric ones in normal conditions and that during ageing, this "switching" from egocentric to allocentric can become impaired and results in problems in navigation[146,147]. Another study showed that old individuals who correctly acquire an egocentric strategy to navigate a virtual maze, performed the same as young individuals but worse when this acquisition became impaired[148], supporting a little bit the idea that egocentric navigation precedes and is less likely to get impaired during ageing in comparison to allocentric, but when this occurs, it prevents the acquisition of visual representations in navigation. Up to this date, there is not a clear answer on why is this a feature of ageing or about the mechanisms that exist behind the decline of allocentric navigation, although some theories have been proposed; the hippocampus is the main brain structure required for the formation of spatial memories, and as I age, the volume of the hippocampus decreases, as well as the number of activated place cells, and affects hippocampal-dependent memory. Nevertheless, for the acquisition of egocentric navigation strategies, some cortical areas are required as well. The impairment in the formation of allocentric frames of references may be due to the fact that the hippocampus gets impaired before cortical structures such as the PFC. Another explanation is that egocentric navigation is preferred during the early stages of life and learning, and only during adolescence and adulthood does allocentric navigation take over. It is thought that abilities that are acquired in the early stages of life and when the brain is more plastic, are somehow more ingrained in the memory than those that are acquired later in life; thus, allocentric navigation is more prone to be damaged than egocentric[149]. One physiological mechanism has been proposed to explain either of those takes on the selection of navigation strategies, and it has been observed in several models that during ageing and in age-related neurological diseases such as AD, the connectivity between the PFC and the hippocampus becomes compromised due to the loss of NMDA receptors, or increased excitability of GABAergic inhibitory pyramidal neurons, so the ability to integrate and switch from egocentric to allocentric becomes impaired. We didn´t observe significative alterations in the basal autophagic flux in the FC; nonetheless, both LC3 II and p62 showed a trend to decrease in the old mice, so this last piece of evidence contributes to the data that I present in this chapter, given that it could be very likely that the upregulation of autophagy by trehalose impairs PFC to DH connectivity by decreasing the signalling through the recycling of the NMDA-R, nonetheless, the exact mechanism that links either autophagy or trehalose to the changes in spatial navigation in the old mice, is still an open question. Overall, I developed a model

in which autophagy can be induced in the ageing dorsal hippocampus that could be used to interrogate spatial memory in the Ndr2 KO mice.

CHAPTER 3


NDR2 DEFICIENCY
PREVENTS AGE-RELATED
SPATIAL MEMORY DECLINE

In previous work from our lab, it was demonstrated that adult Ndr2 KO mice have a lower performance in the Water-Cross Maze, which in some ways is similar to the MWM, only that it is performed in a cross-shaped tank where the platform is hidden in one of the arms. It is considered an easier task than the MWM because the mice only have 2 or 3 choices in which the platform could be located depending on the protocol, and in the same fashion as the MWM, it is a task designed to analyze spatial memory and navigation. Ndr2 KO mice have less accuracy in finding the platform, which indicates a mild impairment in spatial memory; additionally, it was also demonstrated that Ndr2 KO mice have a slower learning curve in the MWM than WT mice. Overall, this proved that Ndr2 is required for correct spatial learning in adult mice. That´s why integrating 2 main observations from our results; that Ndr2 deficiency increases APs in the DH, and that trehalose is able to upregulate autophagy in the ageing DH, I decided to evaluate if there was an age-related impairment in spatial memory in old Ndr2 mice, and whether rescuing the basal autophagic flux with trehalose would have an impact on spatial memory in the mutants.

Old Ndr2 KO mice show impaired autophagy in the DH

We analyzed the basal autophagic flux in the DH of 24-month-old Ndr2 WT and KO mice. Immunohistochemistry against LC3 A/B (Figure 26) and p62 (Figure 27) was carried out. Surprisingly, contrary to what I observed in the young mice in Chapter 1, Old Ndr2 KO mice show less LC3 puncta in the DH compared to the old WT mice, particularly in CA1 and almost significant ($p=0.0573$) in the DG. Something comparable was observed when the staining intensity was analyzed; Ndr2 KO mice showed less LC3 across all brain regions in a more or less homogeneous way. On the other hand, contrary to what was observed with LC3, p62 puncta density in the DH and particularly in DG increased in the KO mice compared to the WT. Additionally, I performed WB analysis with DH samples. Although not significant, I observed the same overall trend: a decrease in LC3 and an increase in p62 protein levels (Figure 28).

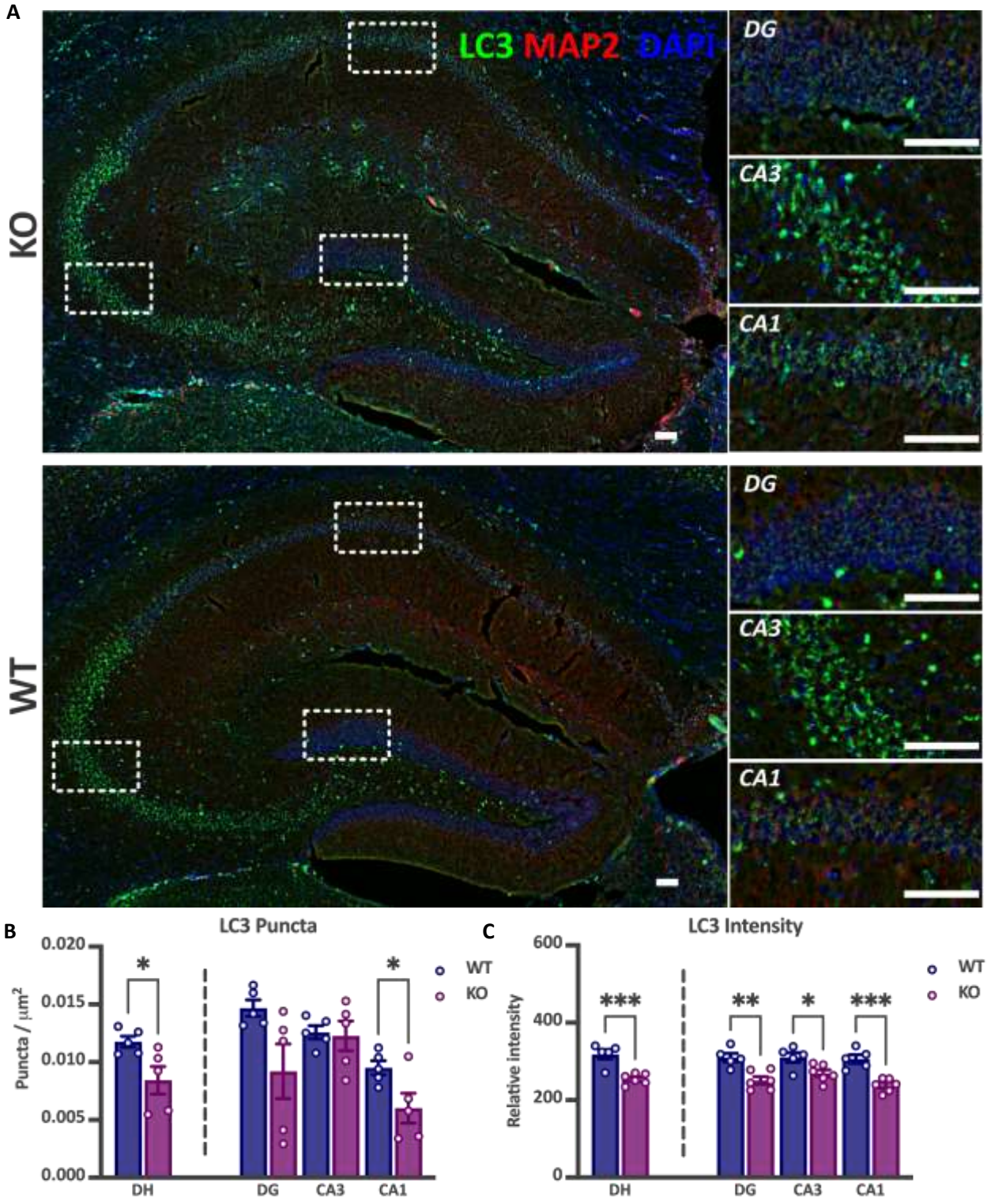


Figure 26. Old Ndr2 KO Mice have decreased LC3 intensity and puncta in the dorsal hippocampus. 30µm Dorsal hippocampus brain slices taken from 24-month-old Ndr2 WT and KO mice were immunostained with LC3 (green) and MAP2 (red) (A). LC3 puncta (B) and LC3 intensity (C) in the DH and in the DG, CA1 and CA3 brain regions were quantified using the spot detector from the software Icy. The bars represent the mean ± S.E.M. Each dot represents the average of 2 brain slices taken from the same mice. Two-tailed unpaired Student´s t-test, *p<0.05, **p<0.005, ***p<0.0005. n=6.

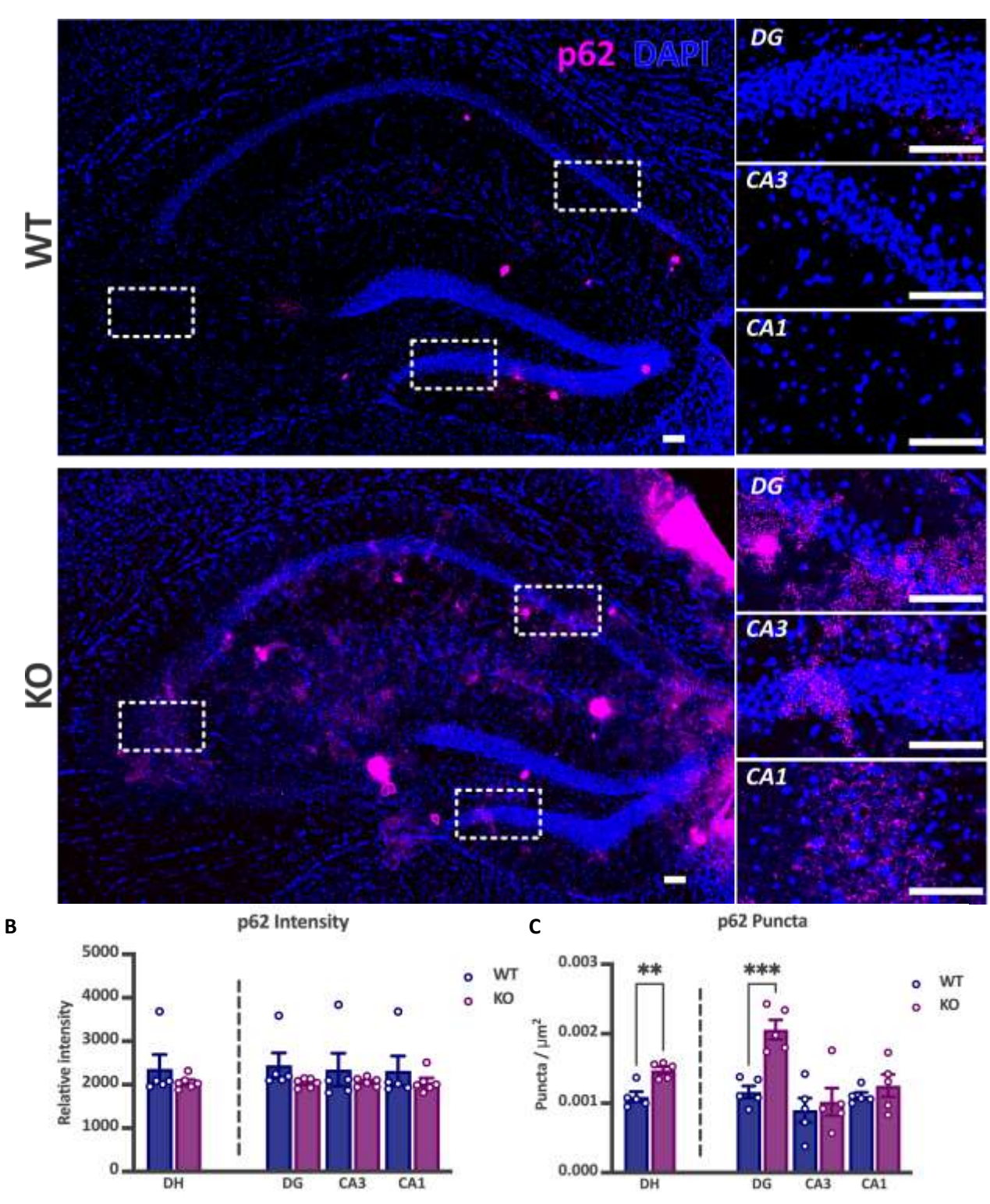


Figure 27. Ndr2 KO Mice have increased p62 puncta in the dorsal hippocampus. 30µm Dorsal hippocampus brain slices taken from 24-month-old Ndr2 WT and KO mice were immunostained with LC3 (green) and MAP2 (red) (A). LC3 puncta (B) and LC3 intensity (C) in the DH and in the DG, CA1 and CA3 brain regions were quantified using the spot detector from the software Icy. The bars represent the mean ± S.E.M. Each dot represents the average of 2 brain slices taken from the same mice. Two-tailed unpaired Student´s t-test, ***p<0.0005. n=5.

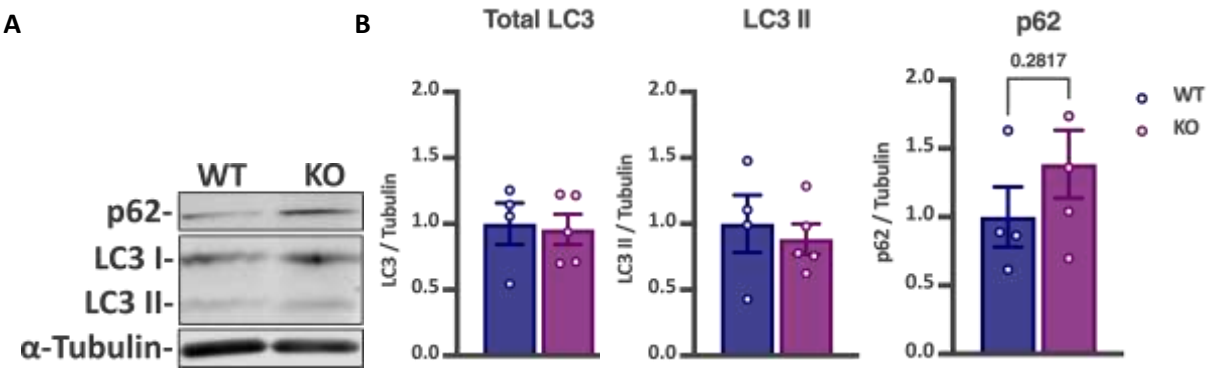
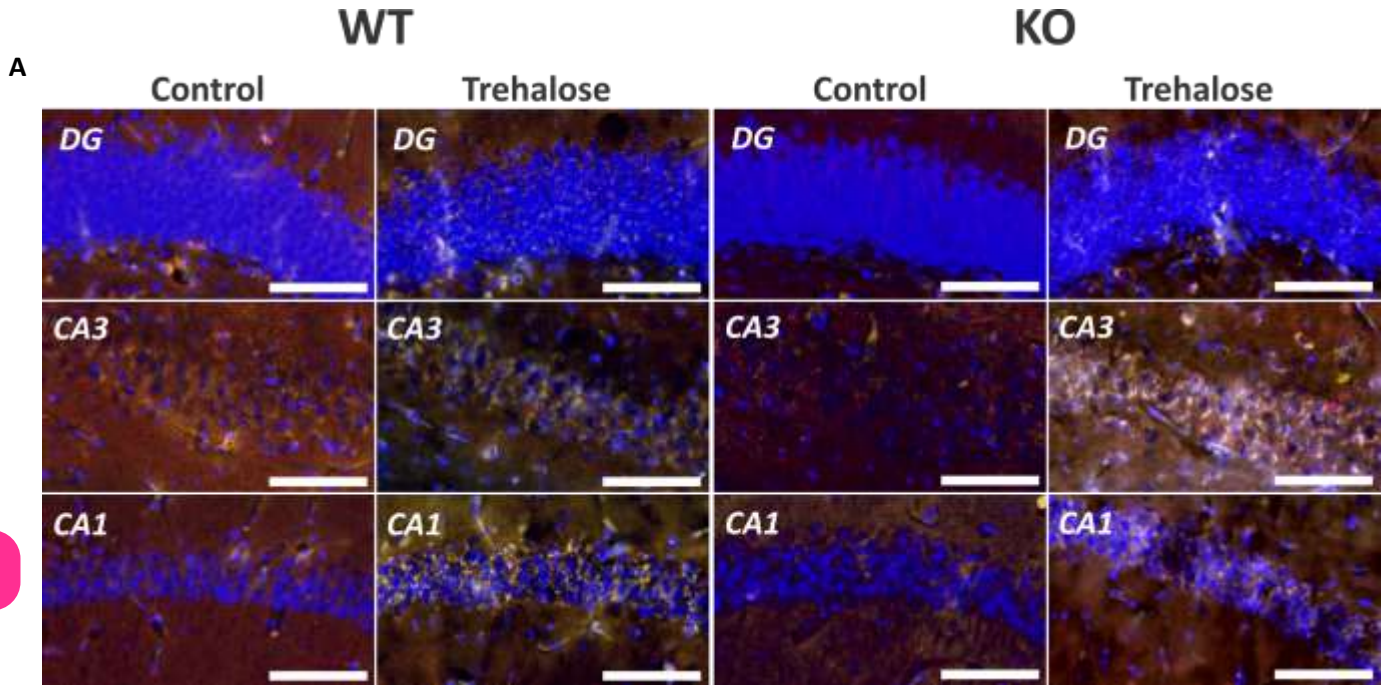


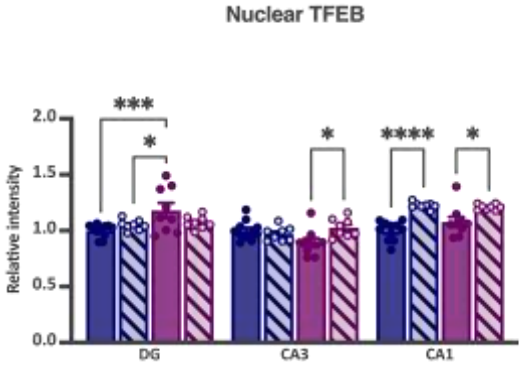
Figure 28. Ndr2 KO Mice have an increased trend of p62 protein levels in the dorsal hippocampus. WB analysis was performed with dorsal hippocampus brain samples taken from 24-month-old Ndr2 WT and KO mice against LC3 and p62 (A). Total LC3, as well as LC3-II and p62 protein levels were quantified (B). The bars represent the mean ± S.E.M. Each dot represents the DH of each mice. *Two-tailed unpaired Student´s t-test*. n=4.

Chronic trehalose administration increases autophagy in the DH of old Ndr2 KO mice.

Next, I treated Old Ndr2 mice with trehalose ad libitum in the drinking water for 10 weeks, and I analyzed the nuclear TFEB signal in the DG, CA3 and CA1 (Figure 29). First, I observed that among the 3 regions analyzed, the TFEB signal in the granular layer of the DG was lower than in the CA3 or CA1 pyramidal layers regardless of genotype and that between the WT and the KO mice, the latter had increased signal. This was surprising because I observed the opposite effect in the CA3, where the TFEB signal was the highest, but the KO mice showed a lower signal than the WT mice. Nevertheless, after the trehalose treatment, the TFEB signal in the pyramidal layer of CA3 was significantly increased regardless of genotype. In the CA1, I observed no differences between the two genotypes but a significant increase after the trehalose treatment.



B



C

Nuclear TFEB			
Source of Variation	Variation	P value	
Region	23,94%	<0,0001	****
Treatment	6,46%	0,0029	**
Genotype	1,87%	0,0925	ns
Region x Treatment	10,48%	<0,0001	****
Region x Genotype	3,90%	0,0146	*
Treatment x Genotype	0,28%	0,5105	ns
Region x Treatment x Genotype	6,89%	0,0008	***
Mice	19,90%		

Figure 29. Chronic trehalose administration increases nuclear TFEB signal in the dorsal hippocampus. 30µm dorsal hippocampus brain slices taken from 24-month-old Ndr2 WT and KO mice after 10 weeks of chronic administration of trehalose were immunostained with TFEB (yellow) and MAP2 (red) (A). The nuclear TFEB signal (B) in the DG, CA1 and CA3 brain regions were quantified and normalized against the whole DG signal. The bars represent the mean ± S.E.M. Each dot represents the average of 2 brain slices taken from the same mice. Three-Way ANOVA. n=8.

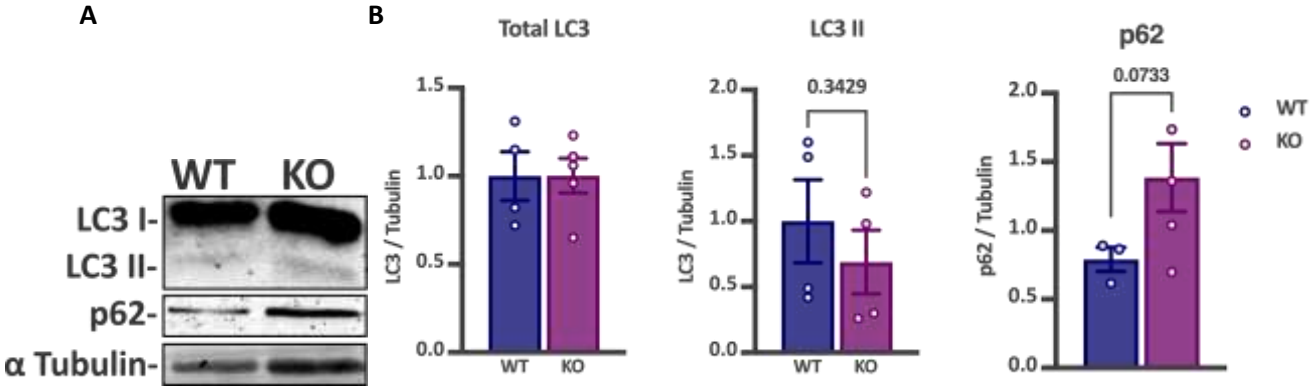


Figure 30. Chronic trehalose treatment mildly decreased LC3 II protein levels in the dorsal hippocampus of old Ndr2 KO mice. WB analysis was performed with dorsal hippocampus brain samples taken from 24-month-old Ndr2 WT and KO mice against LC3 and p62 (A). Total LC3, as well as LC3-II and p62 protein levels were quantified (B). The bars represent the mean ± S.E.M. Each dot represents the DH of each mice. *Two-tailed unpaired Student´s t-test*. n=4.

Pharmacological induction of autophagy with trehalose abolishes the increased spatial memory performance of old Ndr2 KO mice.

Finally, the effect that the pharmacological upregulation of autophagy in the DH had in spatial memory was assessed by subjecting Old Ndr2 WT and KO mice treated with trehalose to training in the MWM plus reversal, spontaneous alternation, and the NOR and NOL tasks (Figure 31). In the MWM task, I observed that there were no differences in the latency to reach the platform located in the southwest quadrant of the maze, either analyzed by training day or by trial. And that though trehalose treatment seemed to stagnate the escape latency at around the 4th day of training, no statistical differences were observed that could be attributed to the genotype or the treatment. Plus, all mice had the same speed when exploring the maze, meaning that they learnt the task in a similar manner and without any impairment (Figure 32). Surprisingly, during the probe trial, when the platform was removed, the KO mice spent more time in the target quadrant than the WT mice, and this increased permanence was abolished upon trehalose treatment (Figure 33). This was the first indication that the Ndr2 mice had better spatial memory than the WT and that by increasing autophagy in the DH with trehalose, spatial memory became impaired. One week after the acquisition, mice underwent reversal learning. The

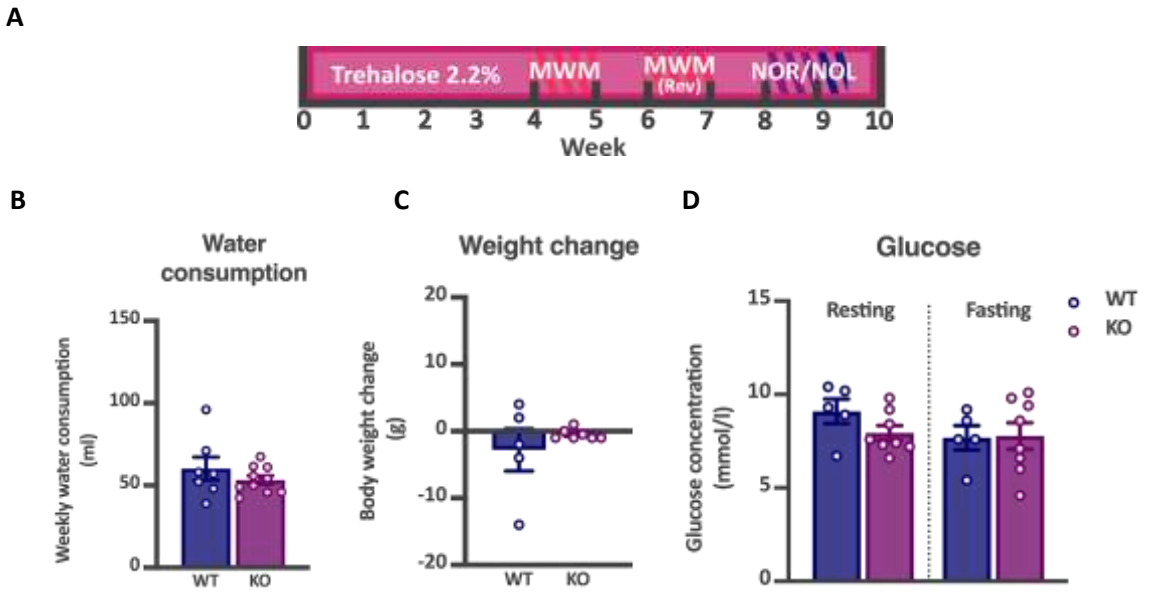
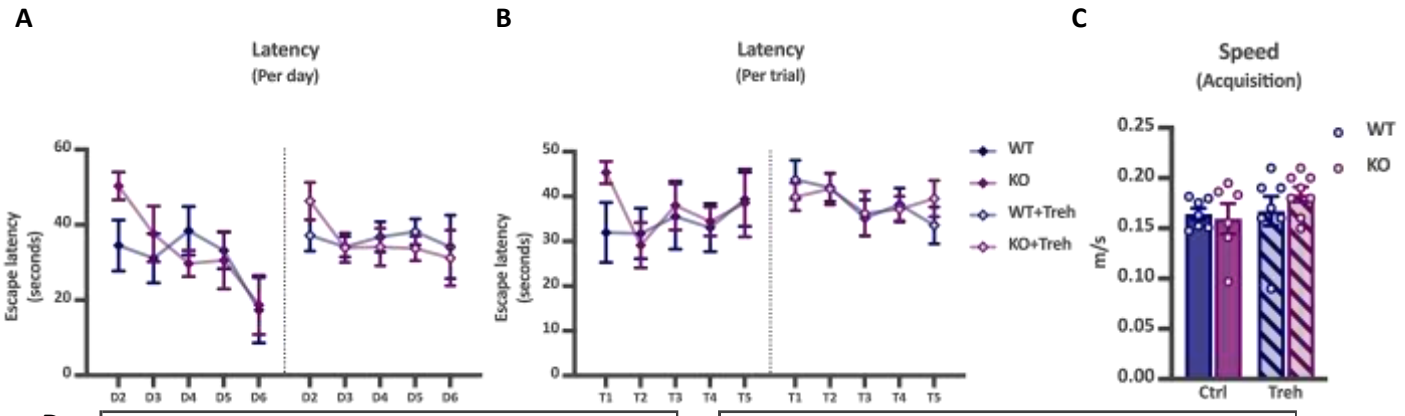


Figure 31. Chronic trehalose treatment doesn't produce changes in weight or glucose levels in the old Ndr2 KO mice. Old (24-months-old) mice were given water *ad libitum* supplemented with trehalose 2.2 % for a period of 10 weeks, during which they were subjected to training in the Morris water maze (MWM) and reversal learning, spontaneous alternation, novel object recognition (NOR) and novel object location (NOL) (A) Water consumption (B) and weight (C) were monitored weekly. *Two-tailed unpaired Student´s t-test*. One day before the end of treatment, glucose was measured in resting conditions and after 8 hours of fasting (D). The bars represent the mean ± S.E.M. Each dot represents each mouse. *Two-way RM-ANOVA* with Bonferroni post-hoc comparison; *p<0.05, **p<0.005. (E). n=6 (WT) & 8 (KO).

platform was changed to the corner-most opposite quadrant (NE), and the latency to find the platform each day and each trial was analyzed (Figure 34). In the training phase, no differences were observed between the WT and the KO mice, showing that the KO mice could re-learn the task. But then, the trehalose-treated mice showed a decrease in latency independently of the genotype, following the same trend that was observed during the acquisition after trehalose treatment. When the latency was analyzed by trial, there were no differences between genotype or treatment, which indicated that the trehalose treatment did not affect short-term spatial memory. In the probe trial when the platform was removed, I observed that in the same way as during the probe trial of the acquisition, Ndr2 KO mice outperformed the WT mice by spending more time close to the target quadrant and also in the same fashion, trehalose treatment decreased that effect in a significant manner. Interestingly, when the permanence only in the NE quadrant was analyzed, I found that the mice that spent less time swimming in the proximity, even below chance level, were the WT mice that underwent trehalose treatment (Figure 35). Next, the spontaneous alternation in the Y maze was analyzed. Mice were placed in a Y-

shape maze and left to explore freely for 8 minutes. The number of alternations, indirect and direct visits was calculated (Figure 36). First, I didn´t observe any significant difference

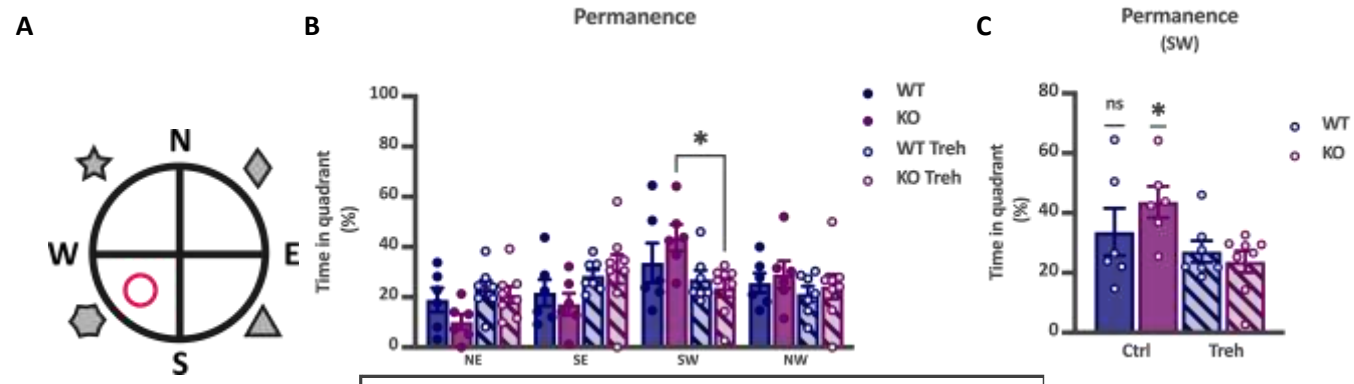


D

Latency per day			
Source of Variation	Variation	P value	
Day	11,73%	0,0011	**
Treatment	1,52%	0,2535	ns
Genotype	0,13%	0,7337	ns
Day x Treatment	3,34%	0,2313	ns
Day x Genotype	4,26%	0,1317	ns
Treatment x Genotype	0,19%	0,6826	ns
Day x Treatment x Genotype	0,58%	0,9113	ns
Mice	25,51%		

Latency per trial			
Source of Variation	Variation	P value	
Trial	1,93%	0,3425	ns
Treatment	1,59%	0,3991	ns
Genotype	0,42%	0,6615	ns
Trial x Treatment	4,10%	0,0537	ns
Trial x Genotype	0,79%	0,7612	ns
Treatment x Genotype	0,25%	0,7382	ns
Trial x Treatment x Genotype	2,86%	0,1599	ns
Mice	49,53%		

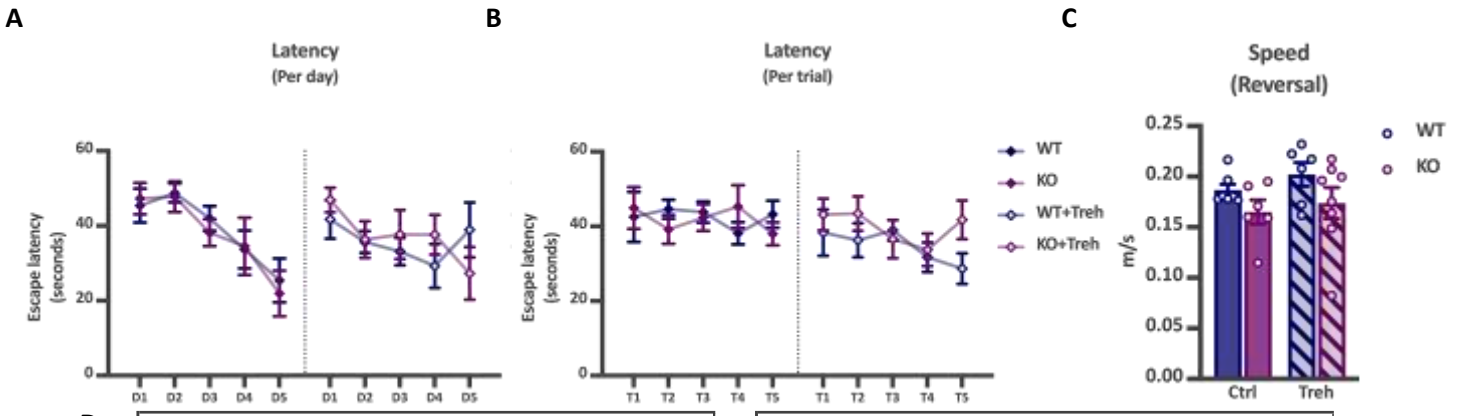
Figure 32. Chronic trehalose treatment doesn't produce changes in the escape latency from the MWM during acquisition in the old Ndr2 KO mice. Old (24-months-old) Ndr2 WT and KO mice were subjected to training in the Morris water maze. The latency to reach the platform on each day (A), each trial (B) *Two-way ANOVA*. The dots represent the mean ± S.E.M. The swimming speed of each mice was analyzed (C). Each dot represents the average of all respective trials of each mouse. *Three-way RM-ANOVA* with Tukey post-hoc comparison (D). n=7 & 6 (WT), 7 & 8 (KO).



D

Permanence			
Source of Variation	Variation	P value	
Quadrant	13,98%	0,0057	**
Treatment	0,00%	0,3156	ns
Genotype	0,00%	0,626	ns
Quadrant x Treatment	13,33%	0,0073	**
Quadrant x Genotype	1,90%	0,6046	ns
Treatment x Genotype	0,00%	0,97	ns
Quadrant x Treatment x Genotype	2,55%	0,4811	ns
Mice	0,00%		

Figure 33. Old Ndr2 mice display increased permanence in the escape quadrant of the MWM that decreases upon chronic trehalose treatment. Old (24-months-old) Ndr2 WT and KO mice were subjected to training in the Morris water maze. In the probe trial, the platform was removed from its location in the SW quadrant (A) and the time spent in each of the quadrants (B) was quantified. The bars represent the mean ± S.E.M. Each dot represents each mouse. *Two-way RM-ANOVA with* Tukey post-hoc comparison, *p<0.05. The time spent in the escape quadrant (SW) was analyzed. *One sample t-test*, theoretical mean=25, *p<0.05. *Three-way RM-ANOVA* with Tukey post-hoc comparison (C). n=7 & 6 (WT), 7 & 8 (KO).



D

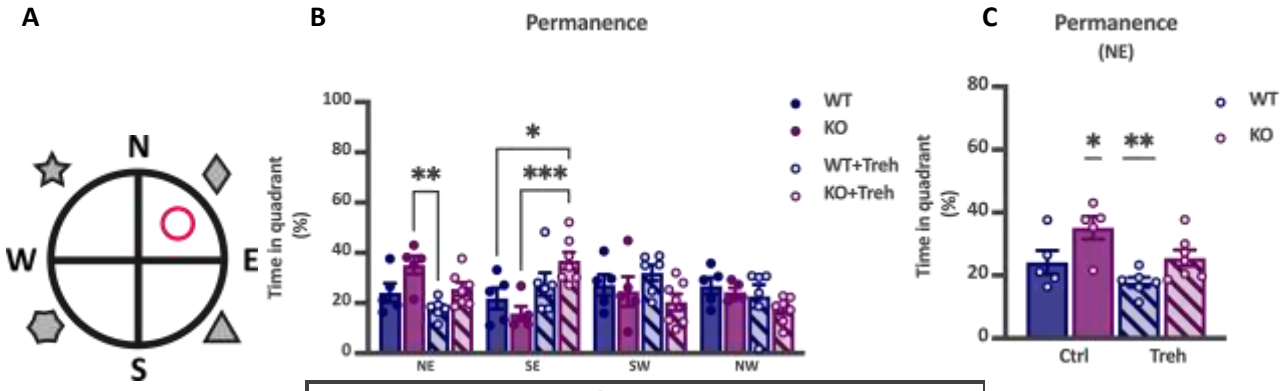
Latency per day

Source of Variation	Variation	P value	
Day	18,81%	<0,0001	****
Treatment	0,55%	0,5288	ns
Genotype	0,00%	0,9633	ns
Day x Treatment	6,42%	0,0402	*
Day x Genotype	2,39%	0,4233	ns
Treatment x Genotype	0,21%	0,6953	ns
Day x Treatment x Genotype	1,13%	0,7612	ns
Mice	25,31%		

Latency per trial

Source of Variation	Variation	P value	
Trial	2,90%	0,2019	ns
Treatment	5,03%	0,1682	ns
Genotype	1,01%	0,5288	ns
Trial x Treatment	1,20%	0,6413	ns
Trial x Genotype	1,20%	0,6404	ns
Treatment x Genotype	1,54%	0,4382	ns
Trial x Treatment x Genotype	3,89%	0,0961	ns
Mice	46,55%		

Figure 34. Chronic trehalose treatment slows the escape latency from the MWM during reversal in the old Ndr2 mice. Old (24-months-old) Ndr2 WT and KO mice were subjected to reversal learning in the Morris water maze. The latency to reach the platform on each day (A), each trial (B). *Two-way ANOVA*. The dots represent the mean ± S.E.M. The swimming speed of each mice was analyzed (C). Each dot represents the average of all respective trials of each mouse. *Three-way RM-ANOVA* with Tukey post-hoc comparison (D). n=7 & 6 (WT), 7 & 8 (KO).



D

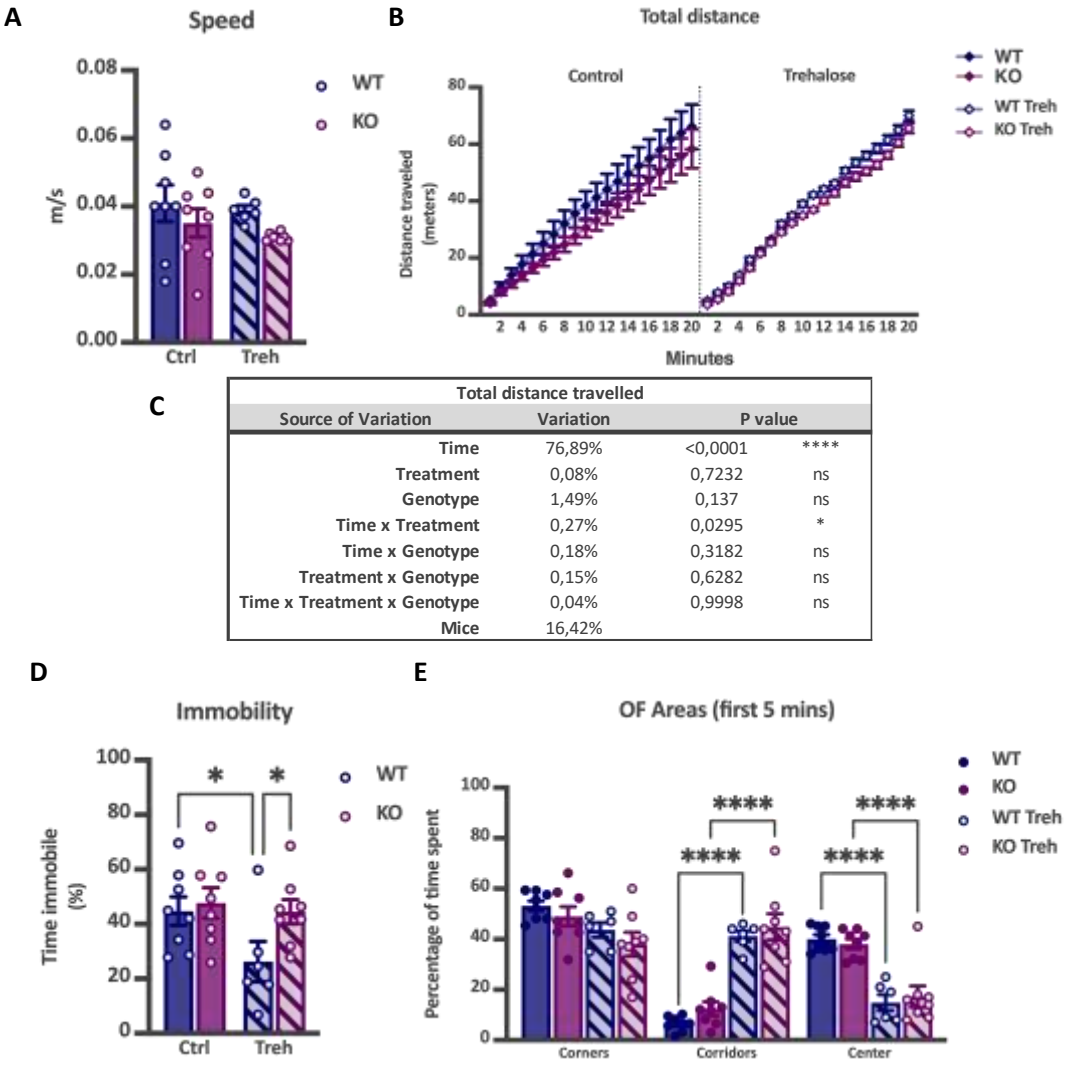
Permanence

Source of Variation	Variation	P value	
Quadrant	1,68%	0,567	ns
Treatment	0,00%	0,9983	ns
Genotype	0,00%	0,9999	ns
Quadrant x Treatment	17,67%	0,0003	***
Quadrant x Genotype	10,22%	0,009	**
Treatment x Genotype	0,00%	0,9999	ns
Quadrant x Treatment x Genotype	5,54%	0,0899	ns
Mice	46,55%		

Figure 35. Old Ndr2 mice display increased permanence in the escape quadrant of the MWM during reversal learning that decreases upon chronic trehalose treatment. Old (24-months-old) Ndr2 WT and KO mice were subjected to re-learning in the Morris water maze. In the probe trial, the platform was removed from its location in the NW quadrant (A) and the time spent in each of the quadrants (B) was quantified. The bars represent the mean ± S.E.M. Each dot represents each mouse. *Two-way RM-ANOVA with* Tukey post-hoc comparison; *p<0.05, **p<0.005, ***p<0.0005. The time spent in the escape quadrant (SW) was analyzed. *One sample t-test*, theoretical mean=25; *p<0.05, **p<0.005. *Three-way RM-ANOVA* with Tukey post-hoc comparison (C). n=7 & 6 (WT), 7 & 8 (KO).

Figure 36. Chronic trehalose treatment increases indirect visits in the spontaneous alternation task in the Y-maze in old mice. Old (24-months-old) Ndr2 WT and KO mice were left to explore a Y-maze freely for 8 minutes. The entries to each arm were recorded, and the number of alternations, indirect visits and direct visits (A) were counted, and the percentage of each kind of sequence was calculated (B). The bars represent the mean ± S.E.M. Each dot represents each mouse. *Two-way ANOVA;* *p<0.05, **p<0.005. n=8 & 8 (WT), 7 & 8 (KO).

between the WT and the KO mice in the number of direct or indirect visits, the only thing that stands out is that most of the KO mice had a higher, but not significant, number of alternations. Surprisingly, those mice that underwent treatment with trehalose displayed an increased percentage of indirect visits and a decreased percentage of direct ones without any changes in the number of successful alternations. The next tested paradigms were the NOR and NOL in the open field. First, mice underwent a habituation period of 20 minutes in the open field box, and the time spent in the corners, the rim and the centre of the maze was analyzed. While there were no evident differences in the overall speed that the animals used to explore, trehalose increased the overall distance travelled regardless of genotype. When the behaviour in the maze was analyzed in the first 5 minutes of the test, I observed that treated WT mice spent less time immobile (60% of body movement halt for at least 2 seconds) than the rest of the groups, additionally, both the WT and KO mice that consumed trehalose, spent less time exploring the centre of the maze and more time in the corridors (Figure 37). 24 hours after the habituation, mice were placed back in the open field, along with 3 different objects (different shapes and different colours) placed close to the corners of the maze, and then, they were left to explore them for 5 minutes, in 3 consecutive trials. For the NOL test, one of the objects was moved across the box to the only empty corner, and the mice I placed back for another 5 minutes to explore freely, and the time spent exploring



Total distance travelled			
Source of Variation	Variation	P value	
Time	76,89%	<0,0001	****
Treatment	0,08%	0,7232	ns
Genotype	1,49%	0,137	ns
Time x Treatment	0,27%	0,0295	*
Time x Genotype	0,18%	0,3182	ns
Treatment x Genotype	0,15%	0,6282	ns
Time x Treatment x Genotype	0,04%	0,9998	ns
Mice	16,42%		

Figure 37. Chronic trehalose treatment decreases time spent in the center of the open field in old mice. Old (24-months-old) Ndr2 WT and KO mice were left to explore freely an open field of 40 cm for 20 minutes. The overall speed (A) was calculated. The bars represent the mean ± S.E.M. Each dot represents each mouse. *Two-way ANOVA;* *p<0.05, **p<0.005. The total distance (B) that the mice travelled was quantified. Each dot represents the accumulated distance of each respective group in a given minute. *Three-way RM-ANOVA* with Tukey post-hoc comparison. The time spent immobile in the first 5 minutes of the test was evaluated (C). *Two-way ANOVA,* *p<0.05. The box was divided into 16 4x4 cm squares (D) and the time on each quadrant was quantified. *Two-way RM-ANOVA with* Tukey post-hoc comparison, ****p<0.0001. n=8 & 8 (WT), 7 & 8 (KO).

the new location (NL) of the object was analyzed, this accounted for the NOL recognition task (Figure 38). I observed that WT mice spent the same amount of time in all 3 objects, which suggests that they didn´t recognize that the object was moved. In contrast, the KO mice spent the greatest time exploring the object that was moved. Concordantly to what was shown before, this mild effect was abolished upon trehalose treatment. As an additional proxy of spatial recognition, the number of investigations

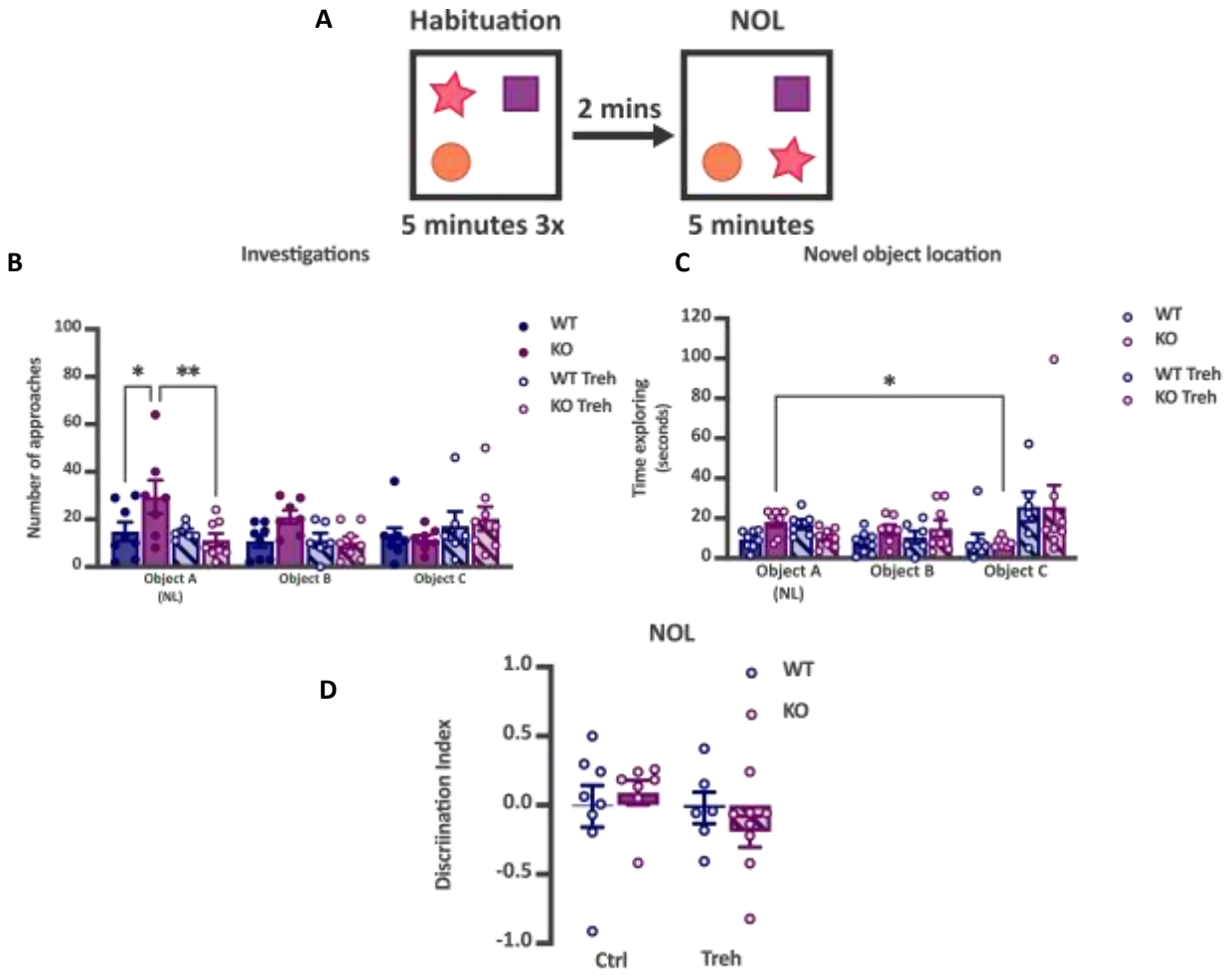


Figure 38. Old Ndr2 mice display better performance in the NOL task that becomes abolished upon chronic trehalose treatment. Old (24-months-old) Ndr2 WT and KO mice were left to explore freely an open field of 40 cm with 3 different objects for 5 minutes 3 times, then one of the objects was moved for to a novel location (NL) (A). The number of investigations (B) and time spent exploring (C) the NL was quantified. The discrimination index was calculated (D). The bars represent the mean ± S.E.M. Each dot represents each mouse. *Two-way RM-ANOVA* with Tukey post-hoc comparison; *p<0.05, **p<0.005. n=8 & 7 (WT), 6 & 8 (KO).

(head-first approaches within 5 cm of the object) was quantified. We also observed an overall preference of the KO mice to investigate the object in the NL compared to the WT mice, which was ablated upon trehalose treatment. The correlation index for the NL was calculated as the difference of time exploring the old locations minus the time exploring the NL divided by the total time exploring. Nonetheless, when the D.I. was calculated, I observed no differences in genotype or treatment, and most of the mice had D.I. close to zero; this suggests that while there was an overall preference to explore the NL, each individual mouse failed to recognize that the object was moved. For the NOR task (Figure 39), one of the stationary objects was replaced with a different one (different colour, different

shape), and the mice were placed back in the box for another 5 minutes. In the same way as with the NOL task, the time spent exploring the novel object (NO) was quantified, as well as the number of investigations. Following the same trend as with the NOL, the KO mice spent more time exploring as well as performing more approaches to the NO compared to the WT mice. Also, in the same fashion, trehalose completely prevented this behaviour. To briefly summarize what I observed in the last figures, the old Ndr2 KO mice display an overall better hippocampal-dependent memory, demonstrated in the MWM plus reversal, NOL and NOR tasks, that is generally abolished in the old Ndr2 KO mice that were administered trehalose.

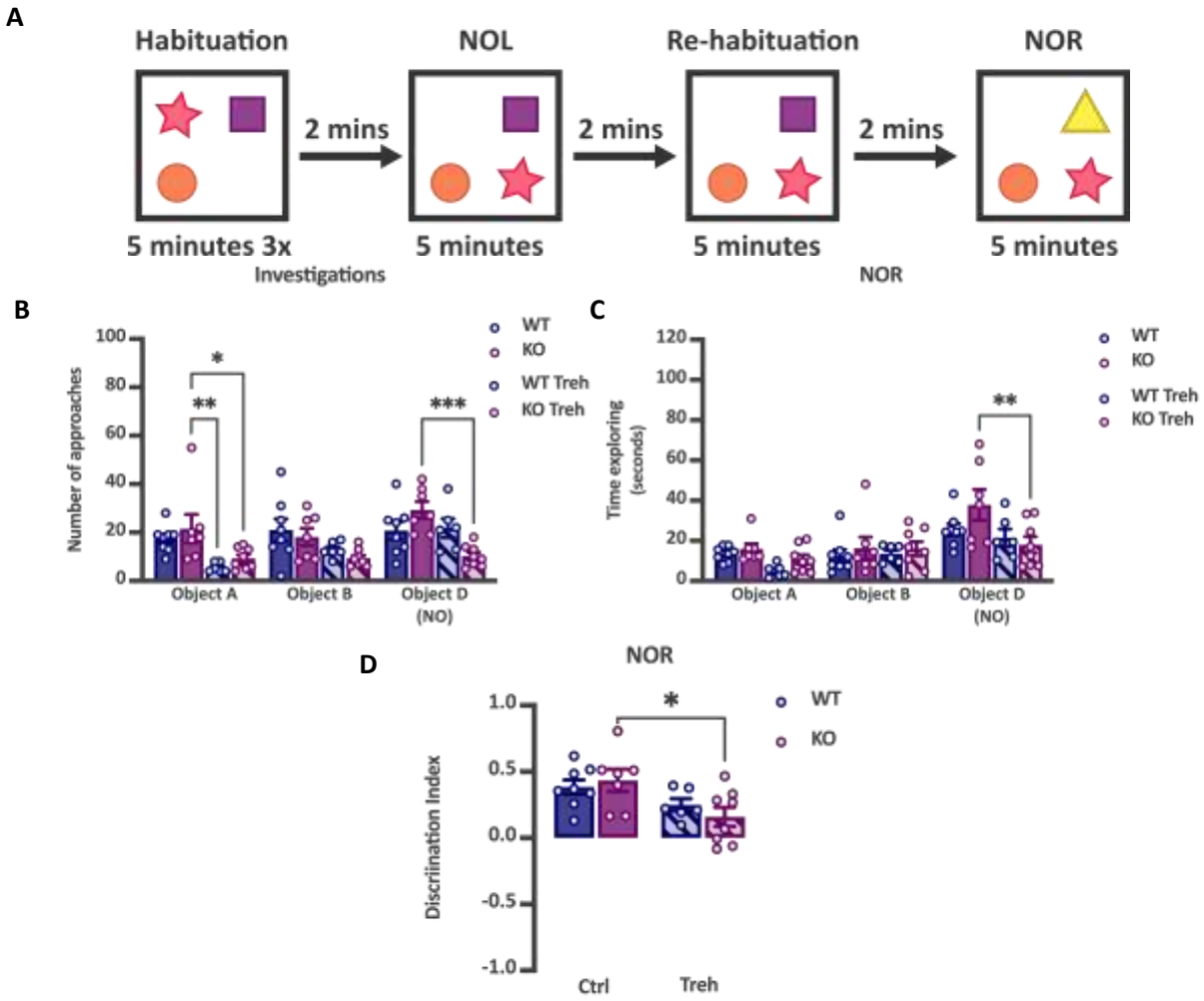
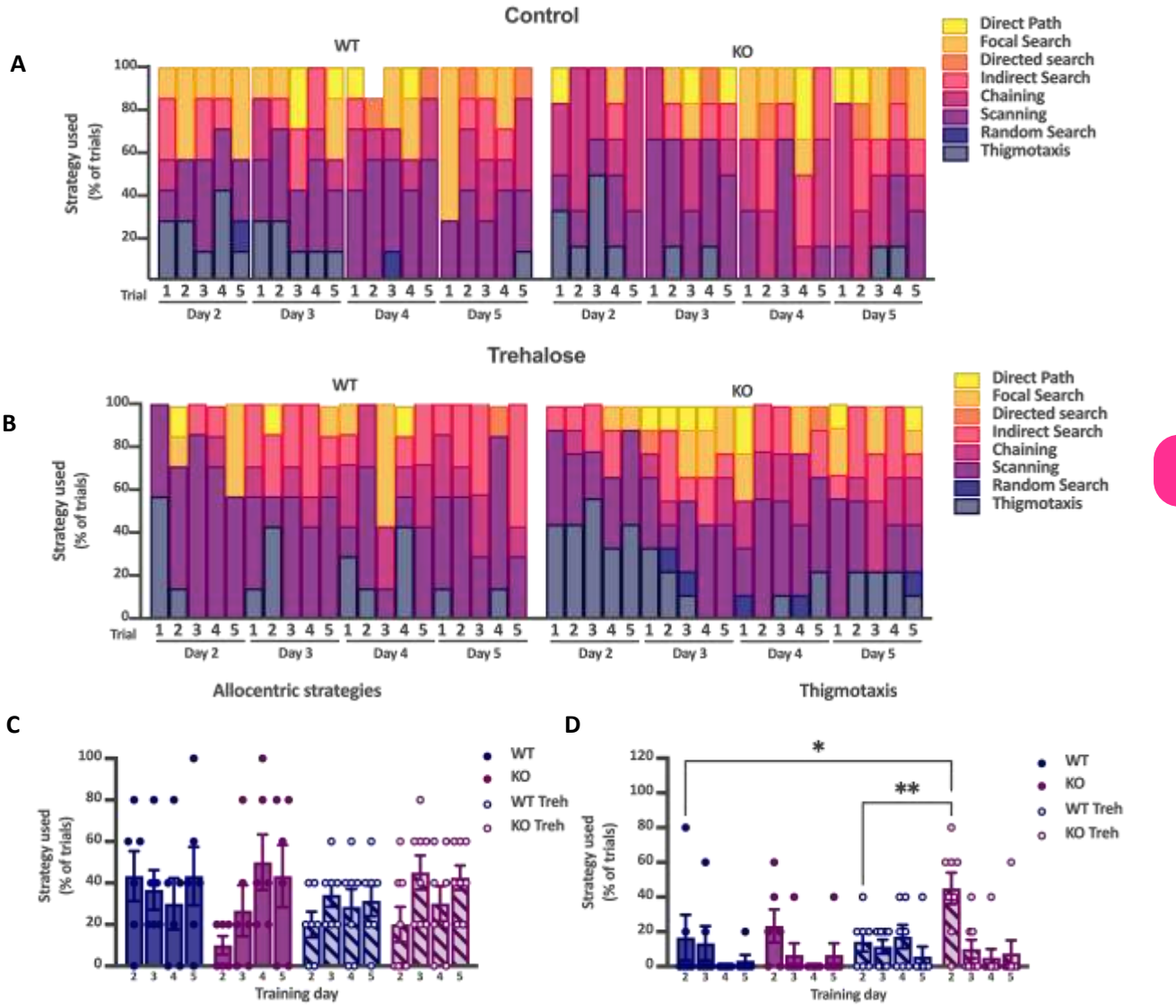


Figure 39. Old Ndr2 mice display better performance in the NOR task than the old WT mice, which becomes abolished upon chronic trehalose treatment. After the NOL test, Old (24-months-old) Ndr2 WT and KO mice were left to explore freely an open field of 40 cm with 3 different objects for 5 minutes, then one of the objects was replaced for a new one (NO) (A). The number of investigations (B) and time spent exploring (C) the NO was quantified. The discrimination index was calculated (D). The bars represent the mean ± S.E.M. Each dot represents each mouse. *Two-way RM-ANOVA* with Tukey post-hoc comparison; *p<0.05, **p<0.005. n=8 & 7 (WT), 6 & 8 (KO).

Pharmacological induction of autophagy with trehalose prevents the increased preference of allocentric navigation strategies of the old Ndr2 KO mice

In order to analyze the possibility that the improved performance in the MWM observed in the KO mice could be due to an increase in the usage of more efficient (allocentric) navigation strategies than the WT mice and whether this behaviour could change after the administration of trehalose, as was established in the chapter before, the exploratory behaviour that the mice used in the learning phase of the acquisition of the MWM was analyzed with Pathfinder (Figure 40). While I didn´t observe any significative differences in the use of allocentric strategies (direct path, focal search, directed search or indirect search), the usage of these strategies increased every training day, reaching its peak between the 4th and 5th day of training, interestingly in the mice treated with trehalose, this behaviour seemed to peak on the 3rd day of training and didn´t increase further on. Contrary to what was observed with the black 6 mice in the chapter before, the preference for chaining didn´t seem to increase in the trehalose-treated animals, but interestingly the usage of thigmotaxis, which is often considered to be even less efficient and an indication that the mice do not actively look for the platform, but only for a way to escape the maze, did seem to be affected by trehalose. When only the usage of thigmotaxis was analyzed on each training day, I observed that the WT and KO mice used thigmotaxis only in the first days of training and very sparsely after that, whereas the mice that took trehalose conserved this preference further on. Moreover, the percentage of mice that used this strategy the most on the second day of training, was among the KO mice that underwent trehalose treatment, indicating that the pharmacological induction of autophagy delayed the usage of strategies aimed at the active search for the platform (random search, scanning, chaining, indirect search, etc...). Interestingly, during the probe trial (Figure 41), all the mice used allocentric navigation in the first 10 seconds of the test, which meant that they learned to locate the platform in this way; nonetheless, after 20 seconds, the usage of allocentric strategies dropped down fast in all the mice, except the KO mice that didn´t take trehalose. This was accompanied by an increase in chaining and scanning, without a strong preference for either; however, some of the WT and the KO mice that took trehalose exhibited thigmotactic navigation, contrary to the control mice, where none of them did it. When the exploratory behaviour used in the reversal learning (Figure 42) was analyzed, something similar to what occurred during the acquisition was observed, all the mice used increased allocentric navigation strategies that increased as the training progressed without differences between the genotype or treatment (albeit less than during the acquisition), that peaked in the last day of training in the WT and KO mice, but seemed to plateau before

in the trehalose treated mice. Again, only some of the mice that were administered trehalose used thigmotaxic navigation during the re-learning. Finally, in the probe trial of the reversal (Figure 43), the trehalose treatment caused a decrease in the use of allocentric navigation in the first 10 and first 20 seconds of the test and an increase in the use of thigmotaxis in the whole trial.

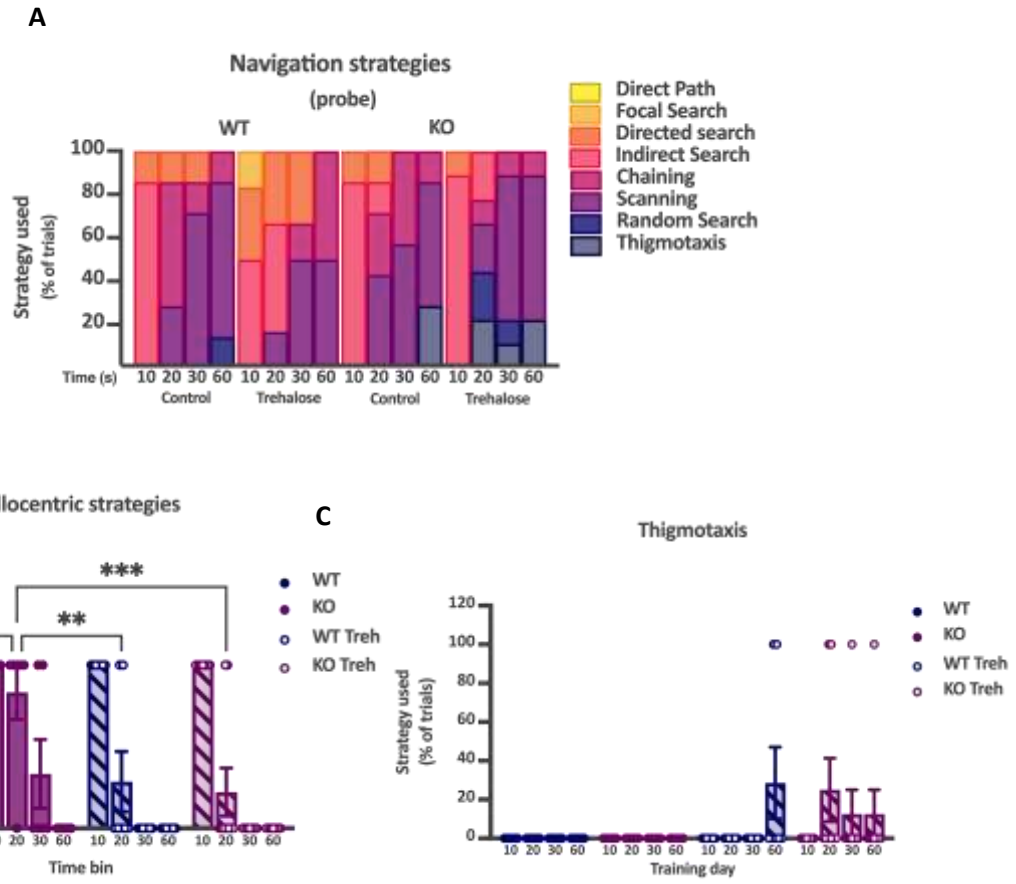


E

Allocentric strategies			
Source of Variation	Variation	P value	
Day	5,94%	0,0428	*
Treatment	0,60%	0,5504	ns
Genotype	0,00%	0,9982	ns
Day x Treatment	1,95%	0,4019	ns
Day x Genotype	4,09%	0,1099	ns
Treatment x Genotype	1,31%	0,3804	ns
Day x Treatment x Genotype	3,54%	0,1541	ns
Mice	37,67%		

Thigmotaxis			
Source of Variation	Variation	P value	
Day	15,22%	0,0001	***
Treatment	2,05%	0,2338	ns
Genotype	0,48%	0,5604	ns
Day x Treatment	1,33%	0,4953	ns
Day x Genotype	5,83%	0,019	*
Treatment x Genotype	0,23%	0,6828	ns
Day x Treatment x Genotype	2,68%	0,1913	ns
Mice	31,46%		

Figure 40. Chronic trehalose treatment mildly reduces the acquisition of allocentric navigation strategies, and increases scanning in old Ndr2 KO mice. Old (24-months-old) Ndr2 WT and KO were trained in the MWM. The navigation strategies used during the learning phase of the acquisition were divided into direct path, focal search, directed search, indirect search, chaining, scanning, random search and thigmotaxis (A). The allocentric strategies (direct path, focal search, directed search and indirect search) were evaluated by each training day (B), as well as the thigmotaxis (C). The bars represent the mean ± S.E.M. Each dot represents each trial. *Two-way RM-ANOVA* with Tukey post-hoc comparison, *p<0.05, **p<0.05. *Three-way RM-ANOVA* with Tukey post-hoc comparison (D). n=7 & 6 (WT), 7 & 8 (KO).

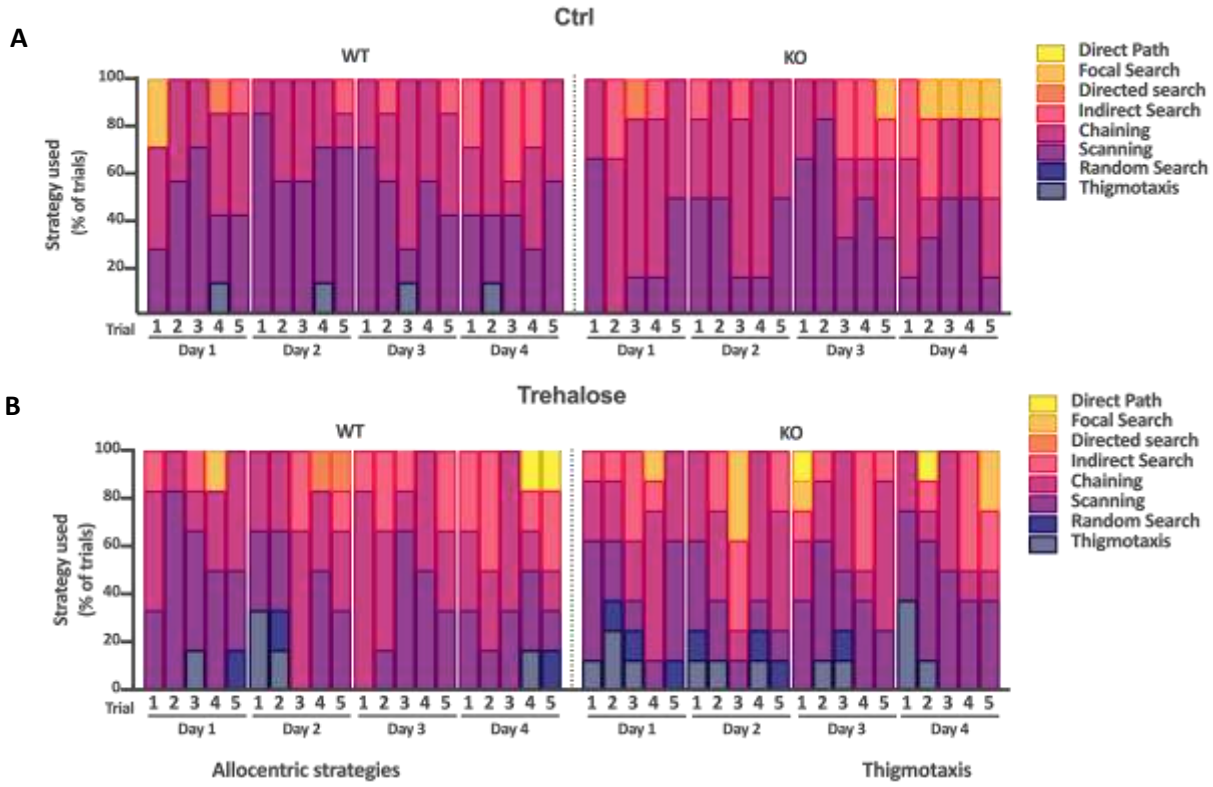


E

Allocentric strategies			
Source of Variation	Variation	P value	
Time	63,24%	<0,0001	****
Treatment	1,48%	0,0491	*
Genotype	1,11%	0,0855	ns
Time x Treatment	1,48%	0,0982	ns
Time x Genotype	1,74%	0,0616	ns
Treatment x Genotype	1,48%	0,0491	*
Time x Treatment x Genotype	2,53%	0,015	*
Mice	8,63%		

Thigmotaxis			
Source of Variation	Variation	P value	
Time	2,71%	0,3928	ns
Treatment	4,53%	0,027	*
Genotype	0,34%	0,5415	ns
Time x Treatment	2,71%	0,3928	ns
Time x Genotype	2,71%	0,3928	ns
Treatment x Genotype	0,34%	0,5415	ns
Time x Treatment x Genotype	2,71%	0,3928	ns
Mice	42,49%		

Figure 41. Old Ndr2 KO mice display increased usage of allocentric navigation that is decreased upon trehalose treatment in the probe trial of the MWM. Old (24-months-old) Ndr2 WT and KO mice were trained in the MWM. The navigation strategies used during the probe trial (A) of the acquisition were analyzed by cumulative time bins (0-10s, 0-20s, 0-30s and 0-60s) (B). The percentage of allocentric strategies (direct path, focal search, directed search and indirect search) used in each time bin was analyzed (C) as well as the thigmotaxis (D). The bars represent the mean ± S.E.M. Each dot represents each mouse. *Two-way RM-ANOVA* with Tukey post-hoc comparison; *p<0.05, **p<0.05, ***p<0.0005, ****p<0.0001. *Three-way RM-ANOVA* with Tukey post-hoc comparison (E). n=7 & 6 (WT), 7 & 8 (KO).

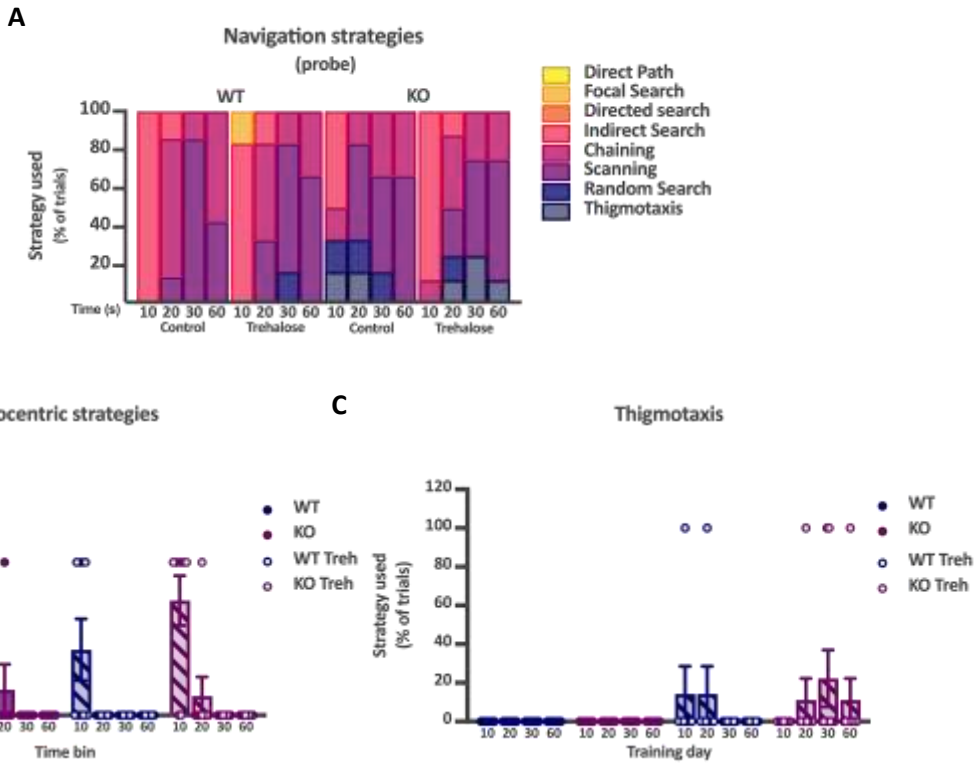


E

Allocentric strategies			
Source of Variation	Variation	P value	
Training day	8,92%	0,0163	*
Treatment	1,37%	0,3408	ns
Genotype	2,14%	0,2354	ns
Training day x Treatment	3,04%	0,2493	ns
Training day x Genotype	0,63%	0,8313	ns
Treatment x Genotype	0,36%	0,6217	ns
Training day x Treatment x Genotype	1,34%	0,6068	ns
Mice	33,22%		

Thigmotaxis			
Source of Variation	Variation	P value	
Day	1,09%	0,7554	ns
Treatment	5,67%	0,0147	*
Genotype	0,45%	0,4838	ns
Day x Treatment	0,91%	0,8028	ns
Day x Genotype	0,50%	0,9077	ns
Treatment x Genotype	1,15%	0,2661	ns
Day x Treatment x Genotype	0,96%	0,7909	ns
Mice	39,03%		

Figure 42. Chronic trehalose treatment increased thigmotactic navigation scanning in old Ndr2 mice. Old (24-months-old) Ndr2 WT and KO were trained in the MWM. The navigation strategies used during the learning phase of the reversal learning of the MWM were divided into direct path, focal search, directed search, indirect search, chaining, scanning, random search and thigmotaxis (A). The allocentric strategies (direct path, focal search, directed search and indirect search) were evaluated by each training day (B), as well as the thigmotaxis (C). The bars represent the mean ± S.E.M. Each dot represents each trial. *Two-way RM-ANOVA* with Tukey post-hoc comparison. *Three-way RM-ANOVA* with Tukey post-hoc comparison (D). n=7 & 6 (WT), 7 & 8 (KO).



D

Allocentric strategies			
Source of Variation	Variation	P value	
Time	62,44%	<0,0001	****
Treatment	2,36%	0,018	*
Genotype	0,44%	0,281	ns
Time x Treatment	4,04%	0,0094	**
Time x Genotype	0,62%	0,5976	ns
Treatment x Genotype	0,44%	0,281	ns
Time x Treatment x Genotype	0,62%	0,5976	ns
Mice	8,38%		

Thigmotaxis			
Source of Variation	Variation	P value	
Time	0,40%	0,9332	ns
Treatment	4,00%	0,0404	*
Genotype	0,19%	0,6525	ns
Time x Treatment	0,40%	0,9332	ns
Time x Genotype	2,30%	0,4808	ns
Treatment x Genotype	0,19%	0,6525	ns
Time x Treatment x Genotype	2,30%	0,4808	ns
Mice	42,49%		

Figure 43. Chronic trehalose treatment reduces the preference for allocentric navigation in the probe trial of the reversal learning of the MWM, and increases thigmotaxis in old Ndr2 mice. Old (24-months-old) Ndr2 WT and KO mice were trained in the MWM. The navigation strategies used during the probe trial (A) of the acquisition were analyzed by cumulative time bins (0-10s, 0-20s, 0-30s and 0-60s) (B). The percentage of allocentric strategies (direct path, focal search, directed search and indirect search) used in each time bin was analyzed (C) as well as the thigmotaxis (D). The bars represent the mean ± S.E.M. Each dot represents each mouse. *Two-way RM-ANOVA* with Tukey post-hoc comparison. *Three-way RM-ANOVA* with Tukey post-hoc comparison (E). n=7 & 6 (WT), 7 & 8 (KO).

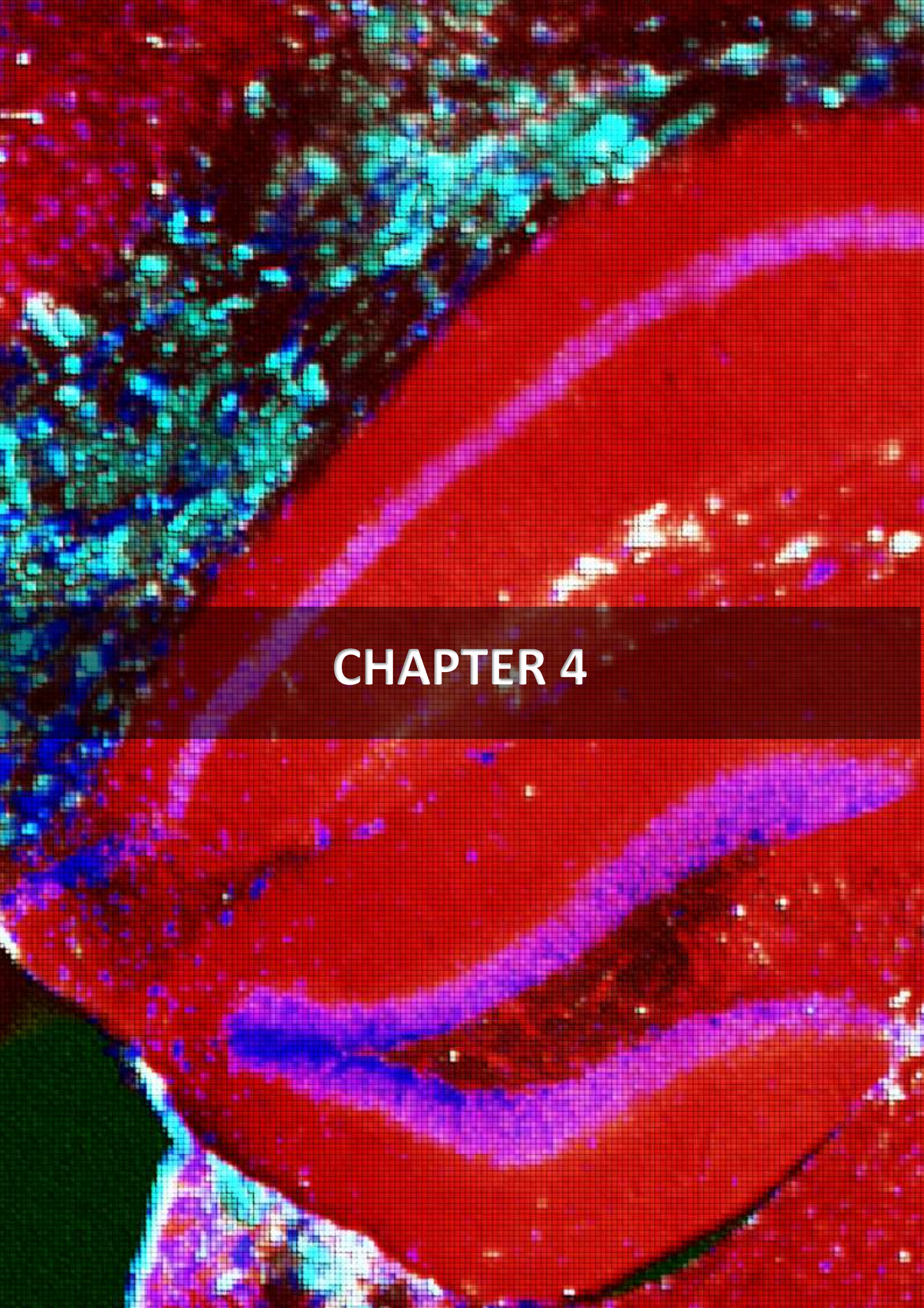
Discussion

In this chapter, I presented results that show that 24-months-old Ndr2 KO mice display impaired autophagy in the dorsal hippocampus, demonstrated by an increase in LC3 signal with less LC3 puncta and an accumulation of p62 puncta compared to WT mice. After the mice were given trehalose in the drinking water for 10 weeks, a strong nuclear translocation of TFEB was observed in the hippocampus's CA3 and CA1 pyramidal layers. This correlated with an increase in LC3 and decreased p62 in the hippocampus. These observations demonstrate that trehalose could rescue, to some extent, the impairment of autophagy observed in the dorsal hippocampus of the old KO mice. Then, hippocampal-dependent memory was evaluated in the MWM plus reversal, spontaneous alternation, the NOR and the NOL paradigms in Old WT and KO mice treated with trehalose. It was demonstrated that KO mice display increased permanence in the escape quadrant of the MWM compared to the WT mice and that upon administration of trehalose, this effect is prevented, both in the acquisition and the reversal learning. Suggesting that old Ndr2 KO mice have better spatial memory than the WT. This was confirmed in the NOL and NOR paradigms. For both the NOL, I observed that the Old KO mice made more approaches and spent more time in the novel location or novel object, respectively, than the WT mice. Concordantly, in both cases, the trehalose treatment abolished this effect. In the spontaneous alternation test, trehalose increased the number of indirect visits while reducing the direct ones, but without changes in the percentage of alternations, regardless of genotype. Though not solely a hippocampal-dependent task, the behaviour in the open field was analyzed, and while there were no differences between the WT and KO mice, trehalose decreased the time spent in the centre of the open field. This suggests that trehalose had other functions besides those observed in spatial memory. Finally, the exploratory behaviour exhibited in the MWM was analyzed. I observed that old KO mice had increased usage of allocentric navigation strategies in the probe trial of the acquisition, compared to the WT mice, and supporting the idea that trehalose impairs spatial memory during ageing, old KO mice treated with trehalose lost this preference. We also observed an overall effect of trehalose in the usage of thigmotactic navigation both in the acquisition and reversal learning and the probe trials that was more accentuated in the probe trial of the acquisition in the old KO mice. Summed up, these results altogether show that Ndr2 KO mice display impaired autophagy in the DH that coincides with a better performance in spatial memory tasks; nonetheless, when autophagy is upregulated in the DH with trehalose, this phenotype is lost.

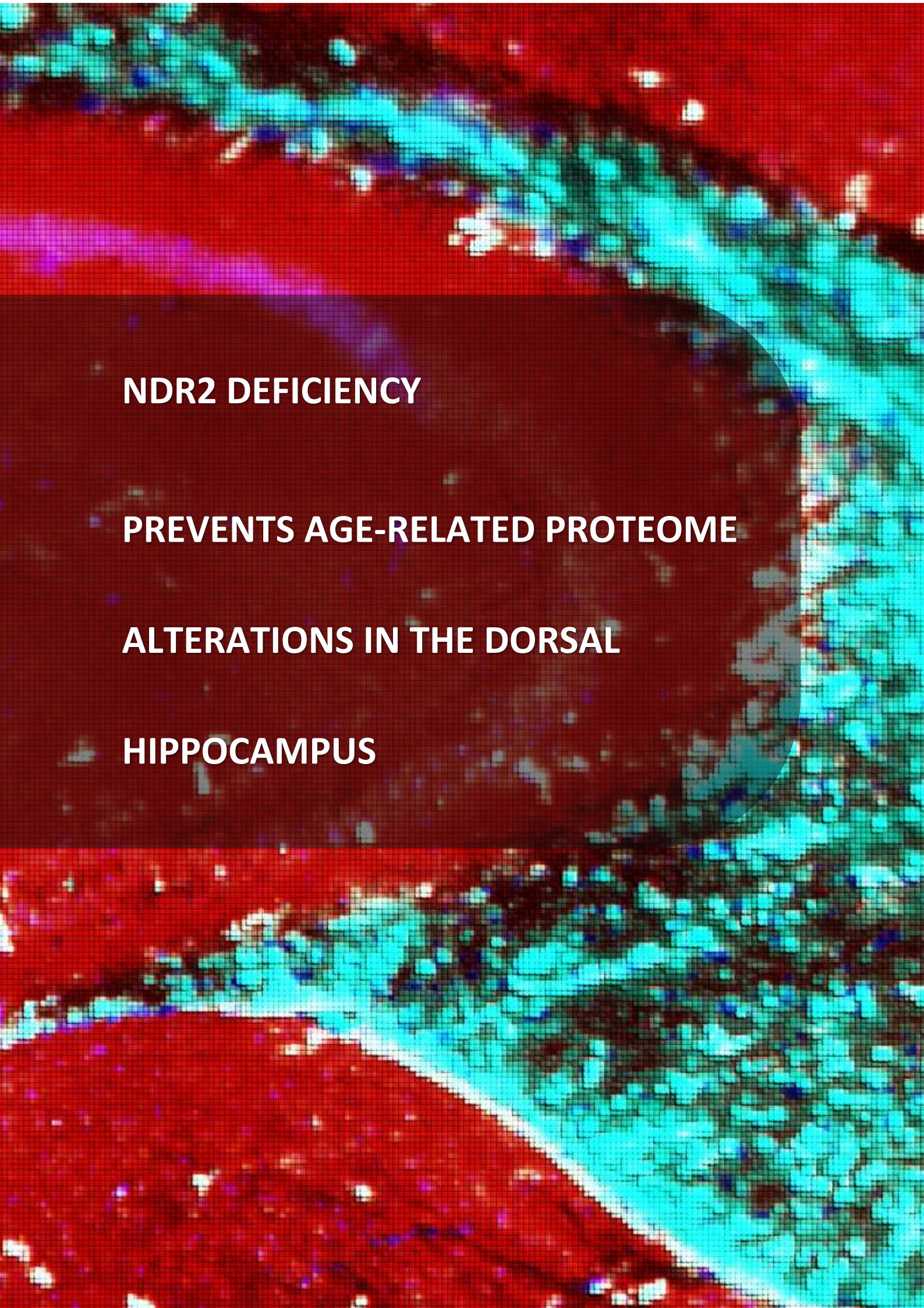
The findings in this chapter show for the first time any evidence that autophagy upregulation in the ageing brain might not always be beneficial and, conversely, that autophagy impairment (in the case of the old Ndr2 KO mice) could actually be beneficial for certain tasks and in certain contexts, like spatial navigation and spatial memory, contradicting what has been published so far. Previous results demonstrated that increasing autophagic flux in the brain by injecting the tat-Beclin 1 peptide prevented memory loss associated with ageing. One possible explanation for this inconsistency is that the tat-Beclin peptide was delivered directly into the dorsal hippocampus, whereas on the other hand, trehalose was delivered in the drinking water, and contrary to the tat-Beclin peptide, which increases autophagy very specifically, trehalose has many more targets other than autophagy that are worth mentioning. In any case, trehalose treatment could increase TFEB protein levels and increase the nuclear translocation of TFEB in the dorsal hippocampus, showing that regardless of the additional effects, trehalose indeed increased TFEB activation. Additionally, the differences observed in the p62 and LC3 puncta in the Old Ndr2 KO mice could be rescued to the WT levels upon trehalose treatment, also indicating that autophagy was increased in the old KO mice. With this in mind, another explanation for the contradictions in cognition after autophagy upregulation is that it is commonly understood that as we age, autophagy becomes downregulated, and this correlates with a decrease in proteostasis, accumulation of damaged organelles, aggregates, etc. But the truth is actually much more complicated than that. As previously discussed in Chapter 2, autophagy regulation becomes less robust when we age, and it gets impaired in many different points of regulation. As it is often mentioned, the result can be decreased aggrephagy which results in the accumulation of damaged biomolecules. Still, it has also been demonstrated that during ageing, selective autophagy is increased and results in the degradation of proteins that are not usually targets of autophagy. One of the most interesting (and relevant examples) is the case of Sirtuin 1 (Sirt1), which is regarded as one of the most important anti-ageing proteins. Its upregulation has been associated with an increase in both lifespan and healthspan among many different model organisms. It is also known that during ageing, Sirt1 levels decrease, and this correlates with the onset of age-related diseases and an increase in intrinsic mortality.[150] Surprisingly, it was recently demonstrated that, in fact, only during ageing and in senescent cells, Sirt1 becomes a target of selective autophagy, which explains the decrease in its protein levels. The mechanism behind that is yet under investigation, but given that Sirt1 is one of the major upstream deacetylases of Ndr2, the possibility that Ndr2 plays a role in a feedback loop that

regulates Sirt1 and in consequence, participates in the targeting or degradation of Sirt1 through autophagy during ageing, can´t be ruled out yet, and fits with the observation that only old, but not young Ndr2 KO mice[151], as was previously demonstrated by our group, have improved spatial memory. This opens up the possibility of analyzing if there are changes in the acetylation of Ndr2 during ageing that could be linked to an aberrant regulation. Another effect that is worth mentioning is that one observed in the open field. The behaviour of rodents in the OF has been comprehensively studied, and it is well-accepted that anxiolytic and locomotor functions can be easily and robustly evaluated in the OF. One of the stereotypical behaviours that has deep roots kindred in the evolution of mice is an increased avoidance of open and bright spaces, attributed to the prevention of predation[152], like what is modelled in the centre areas of the OF. In our data, I show for the first time that trehalose decreased very robustly, and independent of the genotype, the time spent in the centre of the maze in old mice. Mice treated with trehalose spent around 15 to 20 percent of the time there, in harsh contrast with the control mice that spent more than 40% of the time in the centre. This behaviour can´t be explained by a change in locomotor function or increased immobility because it was shown that, in fact, the trehalose-treated mice travelled more distance, albeit just mildly, in the maze over the whole duration of the test. Regarding immobility, trehalose actually increased the percentage of immobility in the WT mice but not the KOs, which could be interpreted under the same light; that trehalose treatment increased anxiety. I can also conclude that this is an age-related effect because it was previously shown in our group that young WT and KO mice show the same behaviour in the OF, regardless of genotype, and that they spent only around 20% of the time in the centre, comparable to old mice that were given trehalose. It is also interesting to note that during ageing, old Ndr2 mice displayed an increased time in the centre, which contradicts some previous efforts to characterize the behaviour in the OF in different ages of B6 mice, where they show a decreased permanence in the centre of the OF that correlates with age[153]. The reason for this difference can´t be completely explained and needs further investigation, but it is known that housing, manipulation and background can easily increase or decrease the anxiolytic behaviour of mice. Given that, it is also of particular interest to explore the effect of trehalose in regions other than the DH, given that I show that trehalose can have a big impact on fear and anxiety behaviours related to the ventral hippocampus and basolateral amygdala (BLA). In that regard, little to nothing is known about how trehalose could affect the fear memory circuits, whether it is due to increased autophagy, ROS or a yet-to-discover unrelated mechanism. One

of the only pieces of evidence that can be extrapolated to our findings is that excessive sucrose consumption (also a disaccharide) in a rat model altered the BLA principal neurons, decreasing the arborization complexity and dendritic length of spines. All and all, the effect of trehalose consumption on the fear and anxiety circuits is worth exploring. Finally, with regard to the changes in exploratory behaviour in the trehalose-treated mice. I observed a very similar effect to what was described in Chapter 2. Trehalose consumption decreased the use of allocentric strategies in old mice. One interesting observation is that during the acquisition and reversal training, both WT and KO mice displayed no preference for any kind of strategy (allocentric or egocentric), there was only an increase in the use of allocentric strategies in the MWM probe trial, so this preference can´t be explained by a difference in learning strategies during the training, and only by an intrinsic difference in the spatial navigation of the KO mice. Surprisingly, I didn´t observe a decrease in the usage of allocentric strategies upon trehalose treatment in any of the 2 genotypes, but what I observed was that although relatively low, there was an increased usage of thigmotaxis, different to what I observed in B6 mice, where the chaining was increased. Thigmotaxis is often considered as the less efficient strategy to search for the platform, and it can even be interpreted as the mice did not learn or were not looking for an escape from the maze. This explains why the trehalose had the greatest negative effect on the Ndr2 KO mice, who displayed an above-average performance in the MWM because it could be that it prevented the acquisition of the task. Another possible explanation, and one that supports the observation of the OF data. Is that a use of thigmotaxis in the MWM (and also the OF) is an increased sign of anxiety[154]. This effect can have 2 consequences; either the mice were too anxious to even learn the task, or they learnt the task, but the increased anxiety prevented the association of the escape platform with an escape from the maze. In conclusion, in this chapter I presented evidence that the spatial memory of the Old Ndr2 KO mice was better than the WT, and I explored one possible mechanism related to autophagy, nonetheless a more in-depth understanding of what is (or are) the mechanisms behind that observation are further investigated in the following chapter.



CHAPTER 4


NDR2 DEFICIENCY
PREVENTS AGE-RELATED PROTEOME
ALTERATIONS IN THE DORSAL
HIPPOCAMPUS

In order to get a deeper understanding of the potential mechanisms of Ndr2 in the ageing hippocampus, why its absence improved memory when compared with old WT mice and even more and to understand why is the behavioural phenotype of the young KO mice different than the old ones; dorsal hippocampus samples from 3 (young) and 24-months-old (old) Ndr2 WT and KO mice were taken from naive mice, and the proteins were identified using a tri-hybrid linear ion-trap quadrupole-Orbitrap mass spectrometer Fusion Lumos ETD.

Ndr2 KO deficiency prevents age-related changes in protein expression in the dorsal hippocampus

After filtering, a total of 37, 182 proteins were identified, and when the fold change compared to the Young WT DH samples was analyzed, I observed that 363 proteins fell under the parameters to be

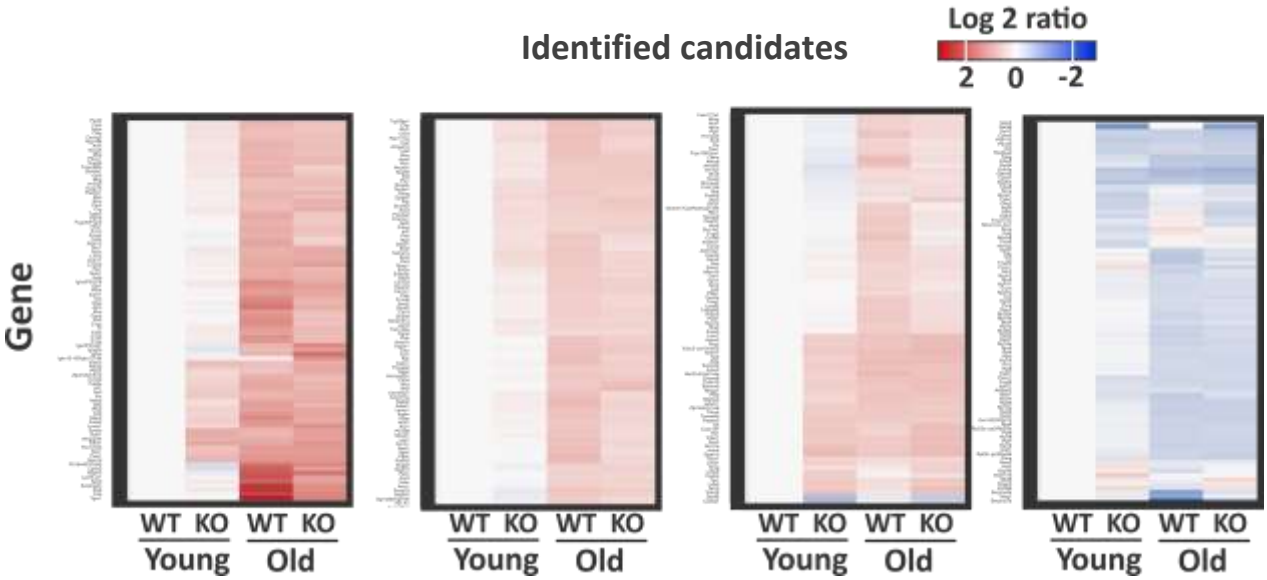


Figure 44. Old mice display age-related changes in the dorsal hippocampus proteome. Dorsal hippocampus samples from young (3-months-old) and old (24-months-old) Ndr2 WT and KO mice were subjected to high resolution liquid chromatography-mass spectrometry in an Orbitrap Exploris Mass spectrometer. The change in protein expression across the different groups was analyzed using the Ggplot2 package for R. The heatmap shows the Log2 fold changes in protein expression normalized against the Young WT mice samples. A protein was considered to be differentially expressed if it had a fold change of >1.5 and a false discovery rate of <0.05. n=4.

considered as "hit" (Figure 44)., and when these hits were clustered using a K-means algorithm (Figure 45), we found 9 different clusters that can be broadly summarized and described as follows:

- Cluster 1: Proteins that are upregulated in all target groups in comparison to the control group (Young WT) and whose increase was higher in the Old mice compared to the young ones **(age upregulation)**.
- Cluster 2: Comprised mostly of proteins that are upregulated in the old mice regardless of genotype, but with a milder change than those in cluster 1, and that also show a mild downregulation in the young KO mice **(mild age upregulation)**.
- Cluster 3: Proteins that are upregulated in the KO mice regardless of age **(KO upregulation)**.
- Cluster 4: Shows the proteins that are upregulated during ageing regardless of genotype, like cluster 1, but whose change is higher in the old WT mice than in the old KO **(strong age upregulation)**.
- Cluster 5. Shows the opposite effect than cluster 1, proteins that are downregulated in the old mice regardless of genotype, and a mild downregulation in the young KO mice (**mild age downregulation**).
- Cluster 6: Proteins that are downregulated in the KO mice, regardless of age (**KO downregulation**).
- Cluster 7: It englobes proteins that are upregulated in all groups but whose upregulation is stronger during ageing (**mild upregulation**).
- Cluster 8: Proteins that decrease in the young KO mice and are strongly upregulated in the old KO mice (**antagonistic KO age effect**).
- Cluster 9: We find the proteins that were upregulated regardless of genotype or age or genotype (**strong upregulation**).

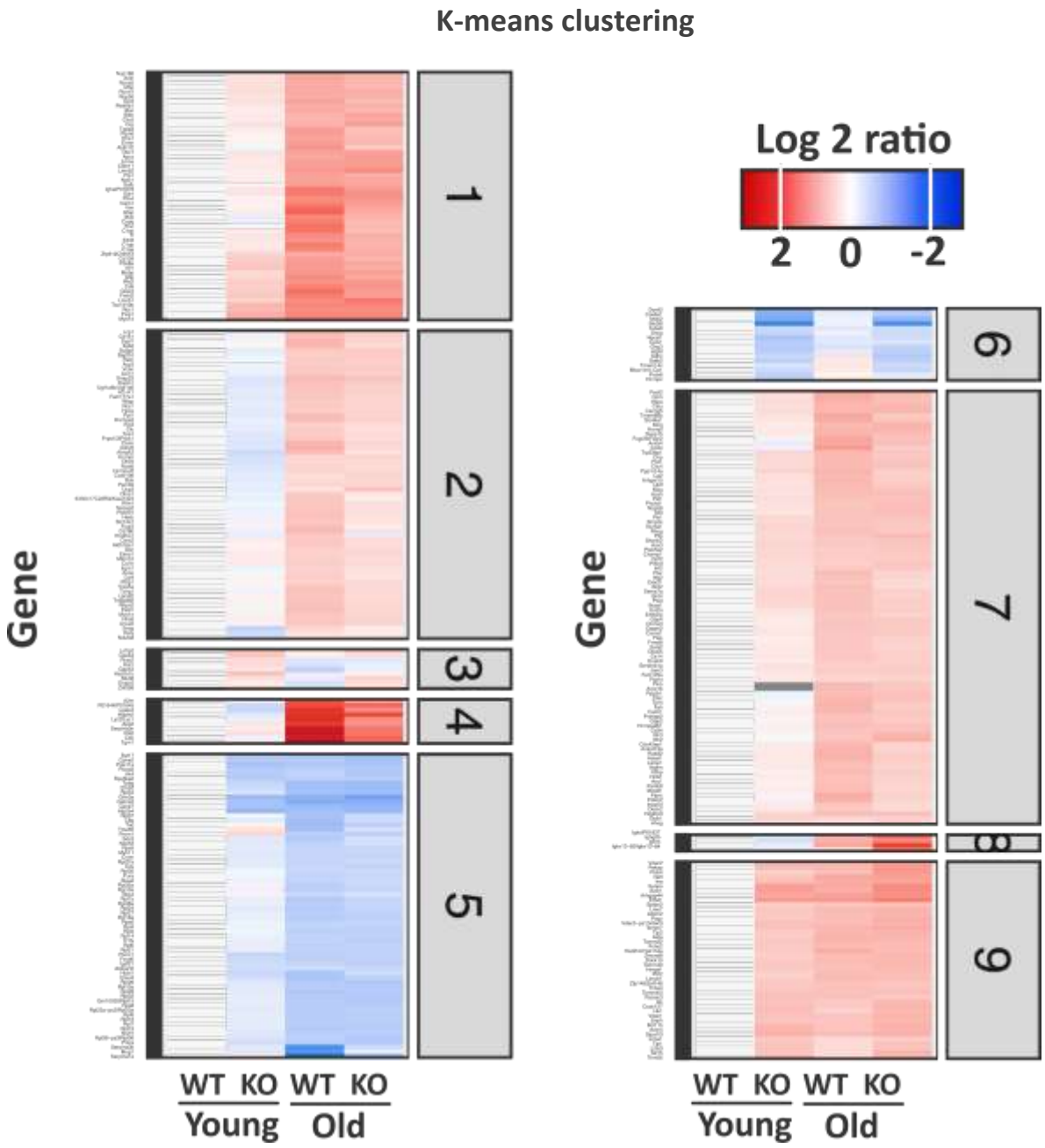


Figure 45. Clusterization of the age-related changes in the dorsal hippocampus proteome, shows an age and phenotype effect. Dorsal hippocampus samples from young (3-months-old) and old (24-months-old) Ndr2 WT and KO mice were subjected to high resolution liquid chromatography-mass spectrometry in an Orbitrap Exploris Mass spectrometer. The proteins were clustered using a K-means algorithm that considered the within groups sum of squares for different cluster numbers. n=4.

Some notable examples from each cluster are the following. Cluster 1: Nuclear lamina A and B, Anillin, Filamin C, Myosin 1D, Glial Fibrillary Acidic Protein and Myelin Basic Protein. Cluster 2: Integrin subunit β2, Versican, SNAP 23, Apolipoprotein E, Neurocalcin Delta, Myosin 1 E and RALY Heterogeneous Nuclear Ribonucleoprotein. Cluster 3: Zinc finger protein 598, Growth associated protein 43, RAS related 2 protein, Lipoprotein lipase, Ndr1. Cluster 4: Galectin 3, Hyaluronan protein 2, Lysozyme 1 and 2, Apolipoprotein D. Cluster 5: Synaptotagmin 17, Ephrin 2, Glutamate receptor 3A, Tenacin C, Ribosomal protein 3, 4, 6, 7, 10a, 17, 18, 19, 21, 24, 27, 35a, 36a. Cluster 6: Cytochrome oxidase 6a, Mob2, Ndr2, α-Tubulin 8, α-Synuclein, Complexin1, ATP synthase 5, Peroxiredoxin 6. Cluster 7: Myelin Oligodendrocyte Glycoprotein, Annexin 4a, CD44, p53, Myelin Associated Glycoprotein, Aggrecan, Interleukin 33, Shank2, Protein Kinase C Delta, Semaforin 7a, Filamin B, Calmodulin, Rabbles 32, Lamp1, α-1 Integrin. Cluster 8: Immunoglobulin Heavy Constant Mu, Immunoglobulin Heavy Constant Gamma 1. Cluster 9: Voltage Dependent Anion Channel 1, Neurofilament Light Chain, α Internexin, Synaptopodin, α -1 Actin, Signal-Induced Proliferation-Associated 1, Voltage Dependent Anion Channel 1 and 3. Something surprising was that in any cluster where we see an age effect, the increase or decrease in the fold change value was always more accentuated in the WT mice than in the KO, suggesting that the absence of Ndr2 might lessen the changes in the protein expression. With that in mind decided to analyze what were the changes in protein expression that occurred during ageing in the WT and the KO mice independently. We plotted the log fold change against the -log 10 of the p-value using the young mice as control from each comparison (Old vs Young WT and Old vs Young KO), creating a volcano plot that highlights the age-related changes in the DH proteome of each genotype (Figure 46). We observed that while in the WT mice, 317 proteins were changed during ageing, in the KO mice, only 98 could be identified as either hits or candidates, showing that in physiological ageing, the absence of Ndr2 prevents age-related changes in the DH proteome. In order to have a more explicit idea of what changes were happening in the old WT mice versus KO during ageing, only the hits and candidates that were not shared during ageing between both genotypes were analyzed. The final list included 236 proteins that changed exclusively in the WT mice and 18 proteins for the KO mice (Figure 47).

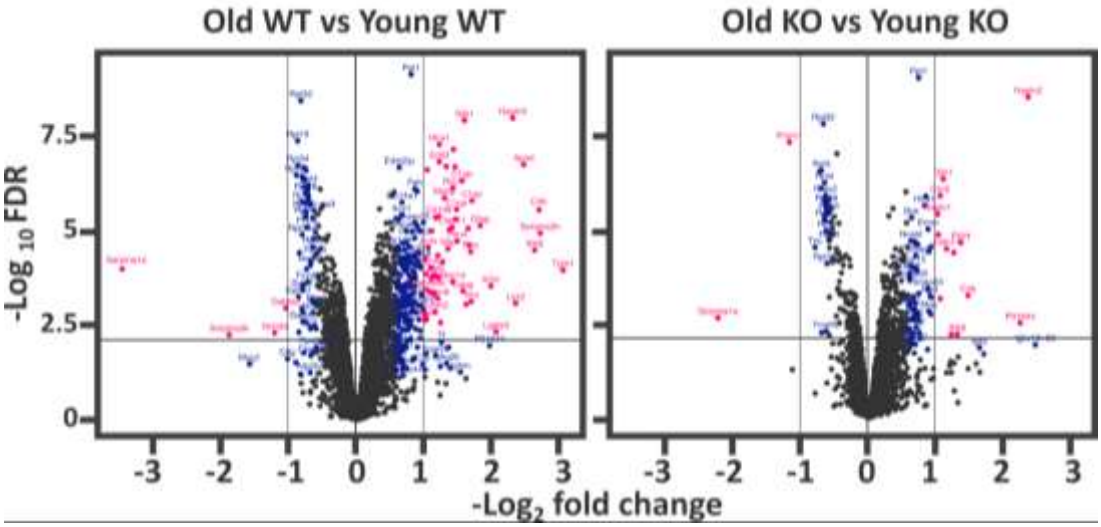


Figure 46. Ndr2 Deficiency prevents age-related changes in the dorsal hippocampus protein expression. The protein expression changes that occurred in the old mice, were normalized against the young mice from the same genotype (age-related changes). The log 2 fold change value was plotted against the -log 10 of the p value. A candidate (blue) was considered if it had a false discovery rate of < 0.05 and a fold change of > 1,5. A hit (pink) was considered when a protein had a false discovery rate of < 0.5 and a fold change > 2. n=4.

Changes during ageing WT

Hit	Gene	Protein ID	Hit	Gene	Protein ID	Hit	Gene	Protein ID	Hit	Gene	Protein ID
H	A2m	Q6GQT1	C	C8g	Q8VCG4	C	Hist2h4	B2RTM0	C	Ppp1r14a	Q91VC7
H	Aif1	O70200	C	Ca14	Q9WVT6	C	Hmga1	P17095	C	Prkcd	P28867
H	Aldh1l2	Q8K009	C	Ca2	P00920	C	Hnrnpa2b1	O88569	C	Prkra	Q9WTX2
H	Anxa4	P97429	C	Cacng5	Q544Q4	C	Hnrnpa3	A2AL12	C	Prps1l3	G3UXL2
H	Capg	Q99LB4	C	Calm1	P62204	C	Icam1	P13597	C	Psmb8	G3UZW8
H	Cd109	A6MDD3	C	Carns1	E9PX09	C	Ighg2b	A0A075B5P3	C	Psmb9	A0A0R4J256
H	Cd44	A2APM1	C	Ccdc127	Q3TC33	C	Ighm	A0A075B5P6	C	Ptbp1	Q8BGJ5
H	Cpm	Q80V42	C	Cck	P09240	C	Igsf21	Q7TNR6	C	Pter	A2AUR3
H	Flnc	D3YW87	C	Cd151	O35566	C	Il33	Q8BVZ5	C	Ptprc	P06800
H	Fmnl2	A2APV2-3	C	Cd180	Q62192	C	Ina	P46660	C	Ptx3	P48759
H	Gabra3	B1AVY2	C	Cdc42ep1	A0A0R4J0S1	C	Inf2	E9QLA5	C	Pura	P42669
H	Glipr2	Q9CYL5	C	Cers2	D3YTM0	C	Inpp5d	Q9ES52	C	Rab32	Q0PD23
H	Grin3a	A2AIR4	C	Champ1	A0A140T8S5	C	Irgm1	Q60766	C	Rftn1	Q6A0D4
H	Igha	A0A075B6A3	C	Clic4	Q543N5	C	Itfg2	Q91WI7	C	Rhog	P84096
H	Lgals3	P16110	C	Clu	Q06890	C	Itgam	Q3U1U4	C	Rhpn2	Q8BWR8
H	Lyz2|Lyz1	P17897	C	Cnp	P16330	C	Itgb2	P11835	C	Rpl23	A0A1
H	Msn	P26041	C	Cpne2	A0A0R4J1D0	C	Itih2	G3X977	C	Rpl24	Q8BP67
H	Myo1d	Q5SYD0	C	Cpne7	Q0VE82	C	Jam3	Q9D8B7	C	Rpl27a	P14115
H	Nup188	Q6ZQH8	C	Crp	P14847	C	Kiaa1324	A2AFS3	C	Rpl35	Q6ZWV7
H	Pbrm1	D3Z1N4	C	Crym	O54983	C	Klc3	D3Z710	C	Rpl36-ps3	A0A140T8K6
H	Pdlim2	Q8R1G6	C	Csf1r	P09581	C	Lamp1	P11438	C	Rpl36a	P83882
H	Psip1	Q99JF8	C	Ctnna3	Q65CL1	C	Lcp1	Q61233	C	Rpl37	Q9D823
H	Ptpn6	P29351	C	Ctsz	Q545I6	C	Lgi4	Q8K1S1	C	Rpl7a	P12970
H	Rcc1	Q6PFB2	C	Daam2	Q80U19	C	Limch1	Q3UH68	C	Rps6ka2	Q9WUT3
H	Serpina3k	A0A0R4J0I1	C	Ddx21	Q9JIK5	C	Lmo7	E9PYI7	C	Rps9	Q6ZWN5
H	Sltm	Q8CH25	C	Dhrs1	Q99L04	C	Lpcat2	Q8BYI6	C	Sdc3	Q64519
H	Tgm1	Q9JLF6	C	Dnah8	Q91XQ0	C	Mag	P20917-2	C	Sema7a	Q9QUR8
H	Tmem88b	Q3TYP4	C	Dock10	E9QM99	C	Micall1	Q8BGT6	C	Serpinb1a	Q9D154
H	Vim	P20152	C	Dock2	Q8C3J5	C	Mtmr10	Q7TPM9	C	Shank2	D3Z5K9
H	Zfp512	Q69Z99	C	Dock5	B2RY04	C	Mug1	P28665	C	Slc14a1	Q8VHL0
C	Ablim2	Q8BL65	C	Dpf2	D3Z5N6	C	Myh11	E9QPE7	C	Slc44a1	A2AMH4
C	Acin1	Q9JIX8	C	Efhd1	Q9D4J1	C	Myo1e	E9Q634	C	Snph	Q80U23-2
C	Actn1	A1BN54	C	Efnb2	P52800	C	Ncoa5	Q91W39	C	Sptbn2	Q68FG2
C	Acy1	Q99JW2	C	Elavl4	A2A9R6	C	Necap2	Q9D1J1	C	Stau2	D3Z6D2
C	Acy3	Q91XE4	C	Elmo1	Q8BPU7	C	Nefl	P08551	C	Synj2	F8WHD8
C	Adcy6	Q01341	C	Erbb2ip	Q80TH2	C	Nop56	Q9D6Z1	C	Synpo	Q8CC35
C	Adssl1	P28650	C	Evi2a	P20934	C	Nop58	Q6DFW4	C	Sypl1	O09117-2
C	Ahnak2	E9PYB0	C	Fah	D3Z2R9	C	Nr2f1	Q32NY6	C	Syt17	Q920M7
C	Ahsg	P29699	C	Fam129a	Q3UW53	C	Numa1	E9Q7G0	C	Tcirg1	Q9JHF5
C	Aida	Q8C4Q6	C	Fcgr2b	A0A0B4J1E6	C	Nxph3	Q544G3	C	Tf	Q921I1
C	Ak4	Q3U489	C	Fcgr3	P08508	C	Osmr	O70458-2	C	Thbs4	B2RTL6
C	Aldh16a1	D3Z0B9	C	Fcho2	F6RG68	C	Pacsin3	Q543N7	C	Tjp2	Q9Z0U1
C	Aldoart2	A6ZI47	C	Flnb	Q80X90	C	Padi2	Q08642	C	Tnfaip8l2	Q9D8Y7
C	Ankrd13a	Q80UP5	C	Frmd8	Q3UFK8	C	Pakap	F7AA26	C	Tomm22	Q9CPQ3
C	Anxa5	P48036	C	Fxyd6	Q9D164	C	Pald1	P70261	C	Tomm40	Q9QYA2
C	Aox3	G3X982	C	Gap43	P06837	C	Pde11a	P0C1Q2	C	Tomm40l	Q9CZR3
C	Apcs	P12246	C	Gbp2	Q9Z0E6	C	Phf2	Q9WTU0	C	Tomm7	Q9D173
C	Apoe	P08226	C	Gbp4	Q61107	C	Phyhd1	A0A0A0MQG9	C	Tpbg	Q9Z0L0
C	Arhgap44	Q5SSM3	C	Gjc2	Q8BQU6	C	Plcl1	Q3USB7	C	Trp53bp1	A2AU89
C	Arhgef10	Q8C033	C	Gk	Q64516	C	Plcxd3	G3X9A7	C	Ubtf	A2AWT5
C	Arpc1b	Q91Z25	C	Gsn	P13020	C	Pld1	D6RH77	C	Ugt1a6b	K9J7B2
C	Aspa	Q8R3P0	C	H2-K1	P01901	C	Plek	Q5F271	C	Vdac1	Q60932-2
C	Atp2b4	E9Q828	C	Hat1	A2ATU9	C	Plekha2	Q9ERS5	C	Vdac2	Q60930
C	Bbs4	A6H669	C	Hbbt1	A8DUK4	C	Plekhf1	Q3TB82	C	Vdac3	Q60931
C	Bcas1	Q80YN3	C	Hcls1	P49710	C	Plgrkt	Q9D3P8	C	Vwa5a	Q99KC8
C	Bcl11b	Q99PV8-2	C	Hdgfrp3	Q9JMG7	C	Pllp	Q9DCU2	C	Wtap	E0CYH0
C	Bmp2k	Q91Z96	C	Hexb	P20060	C	Pls1	Q3V0K9	C	Zfp148	Q548L0
C	Brd2	Q7JJ13	C	Hhatl	Q9D1G3	C	Plxnc1	Q9QZC2	C	Zmynd8	A2A482
C	Brd4	Q3UH70	C	Hist1h2bm	B2RTK3	C	Pogz	B9EIG6	C	Znf598	Q80YR4

H Hit
C Candidate

Changes during ageing KO

Hit	Gene	Protein ID
C	Rras2	P62071
C	Bok	O35425
C	H1fx	Q80ZM5
C	Ncald	Q91X97
C	Ucp3	B2RTM2
C	Dhx9	E9QNN1
C	Ndufs8	Q5M9P5
C	Palm	Q9Z0P4
C	Hnrnpul2	Q00PI9
C	Raly	A2AU61
C	Ccdc136	Q3TVA9-2
C	Hnrnpc	Q9Z204-2
C	Hist1h1b	P43276
C	Hnrnpr	Q8VHM5
C	Sept10	Q8C650-2
C	Sncg	Q5GQ64
C	Igkv13-85	A0A075B666

Figure 47. Old WT mice have more unique age-related protein alterations during ageing than the KO mice. List of the proteins that change during ageing in the WT but not the KO mice (A). Proteins that change during ageing in the KO but not the WT mice (B).

Ndr2 KO deficiency prevents age-related changes in inflammation, translation and mitochondrial pathways in the dorsal hippocampus

Then, a pathway enrichment analysis was done with g: Profiler using gene ontology terms for "biological processes" (BP), "cellular compartment" (CC) and "molecular functions" (MF) from the Gene Ontology Consortium (GO). Then, the protein network changes were evaluated using STRING (v 11.5) and visualized in Cytoskape, using the GO terms, as well as those from the Kyoto Encyclopedia of Genes and

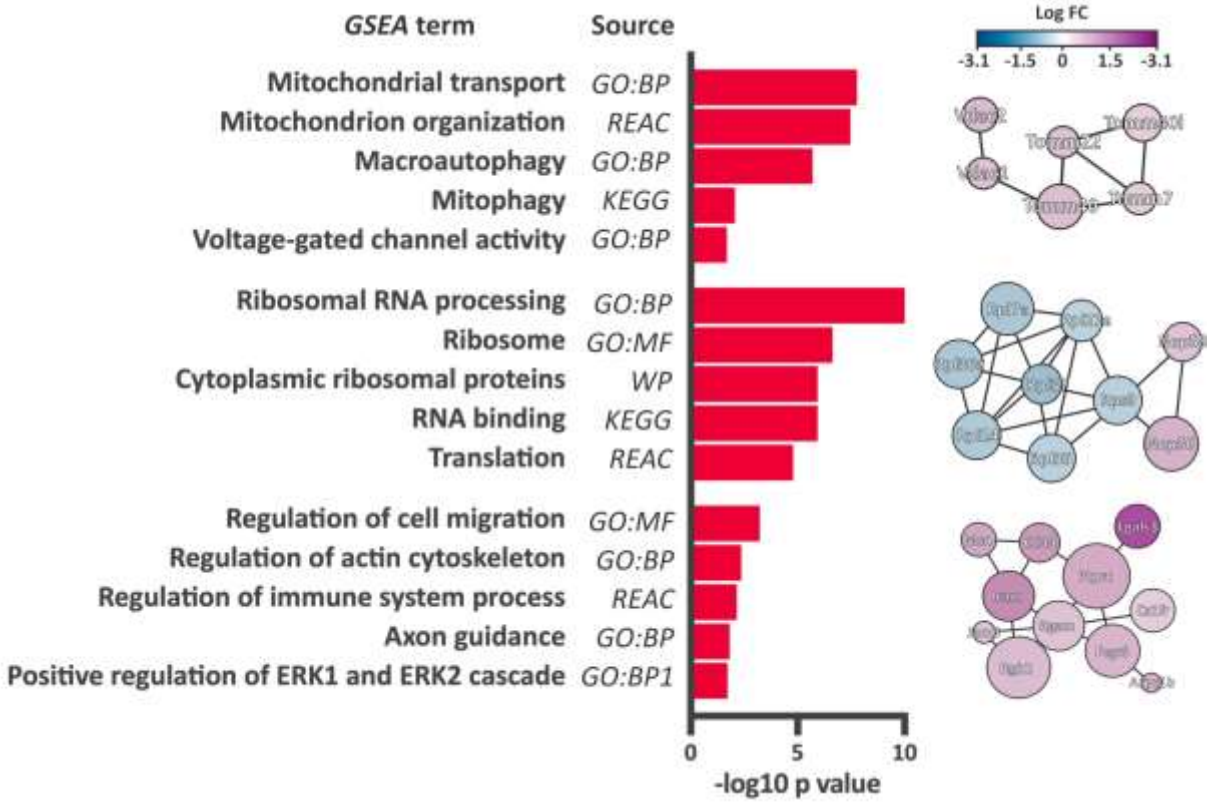


Figure 48. Ndr2 deficiency prevents age-related alterations in mitochondrial, ribosomal, and inflammation pathways in the dorsal hippocampus. The protein network changes that occur uniquely during ageing (young vs old) in the WT but not in the KO mice were evaluated with a gene pathway enrichment analysis using g:Profiler with gene ontology terms for "biological processes" (BP), "cellular compartment" (CC) and "molecular functions" (MF) from the Gene Ontology Consortium (GO) as well as pathways from the Kyoto encyclopedia of genes and genomes (KEGG) and Reactome. The protein networks were visualized using STRING (v 11.5) and Cytoskape (v.3.9.1).

Genomes (KEGG) and Reactome (Figure 48). The pathways and protein interaction networks that were the most changed in the old WT mice but not the KO (14 in total) were mostly related to inflammation,

mitochondrial function and protein translation; surprisingly, we only found one pathway in the KO mice that was related to mRNA splicing. The pathway that involved the highest number of proteins in the WT included Arpc1b, Cd44, Csf1r, Fcgr3, Icam1, Itgam, Itgb2, Jam3, Lgals3, Msn and Ptprc. These proteins altogether are mostly related to the regulation of locomotion, migration and cell adhesion and in the nervous system, they are also involved in axon guidance, nervous system development and integrin signalling. The second biggest pathway to be altered in the WT ageing was the one that involved Nop56, Nop58, Rpl24, Rpl27a, Rpl35, Rpl36a, Rpl37, Rpl7a and Rps9. Most of these proteins are ribosomal proteins from the large ribosomal subunit or other structural constituents of the ribosome. Therefore, they are involved in ribosomal biogenesis and rRNA processing. The third most changed pathway included the mitochondrial proteins Tomm22, Tomm40, Tomm40l, Tomm7, Vdac1 and Vdac2, and was related to mitochondrial functions and mitochondrial transport. Interestingly, TOMM40 and Vdac1 have also been implicated in ALS and Vdac 1 and 2 in mitophagy. One last noteworthy mention is that we also observed that some components of the Rac and Ras signalling were altered (Dock2, Elmo1, Rhog), which are also involved in phago and endocytosis. In all these pathways, the protein whose expression changed the most during ageing was Galectin 3 (lgals3). It is a galactoside-binding lectin that is mainly localized in the nucleus but shuttles out to the cytoplasm and can be secreted. It is mostly expressed in immune cells such as eosinophils, mast cells, T and B cells, etc., but in the brain, it can be found in the microglia and the sensory neurons. Among its functions, it stands out that it is involved in inflammation and fibrosis by interacting with extracellular matrix proteoglycans as a signalling molecule. In that context, it has been linked to the progression of several diseases such as cancer, heart disease, kidney disease, and even neurodegenerative disorders like Huntington´s disease in such a way that it has been proposed to serve as a marker for the severity of these diseases. But more relevant to our work is that in the context of memory, it has been demonstrated that overexpression of galectin 3 (Gal3) negatively regulates hippocampal-dependent memory formation through the inhibition of the a-1 integrin subunit and inhibition of LTP; for this reason, is why Gal3 is a very promising target that could explain the increased spatial memory performance observed in the old KO mice. We performed IHCC against Gal3 in brain slices from old WT and KO mice (Figure 49). we observed that while in the WT brains, there were glial-like cells that expressed Galectin 3 across the whole hippocampus, the Gal3 positive cells were from scarce to null in the KO mice, confirming the data obtained from the Mass spec analysis. Additionally, we observed a very high expression of Gal3 outside the hippocampus, in the alveus, which

is part of the fornix system and is comprised mainly of the efferent fibres of the pyramidal cells in the CA1.

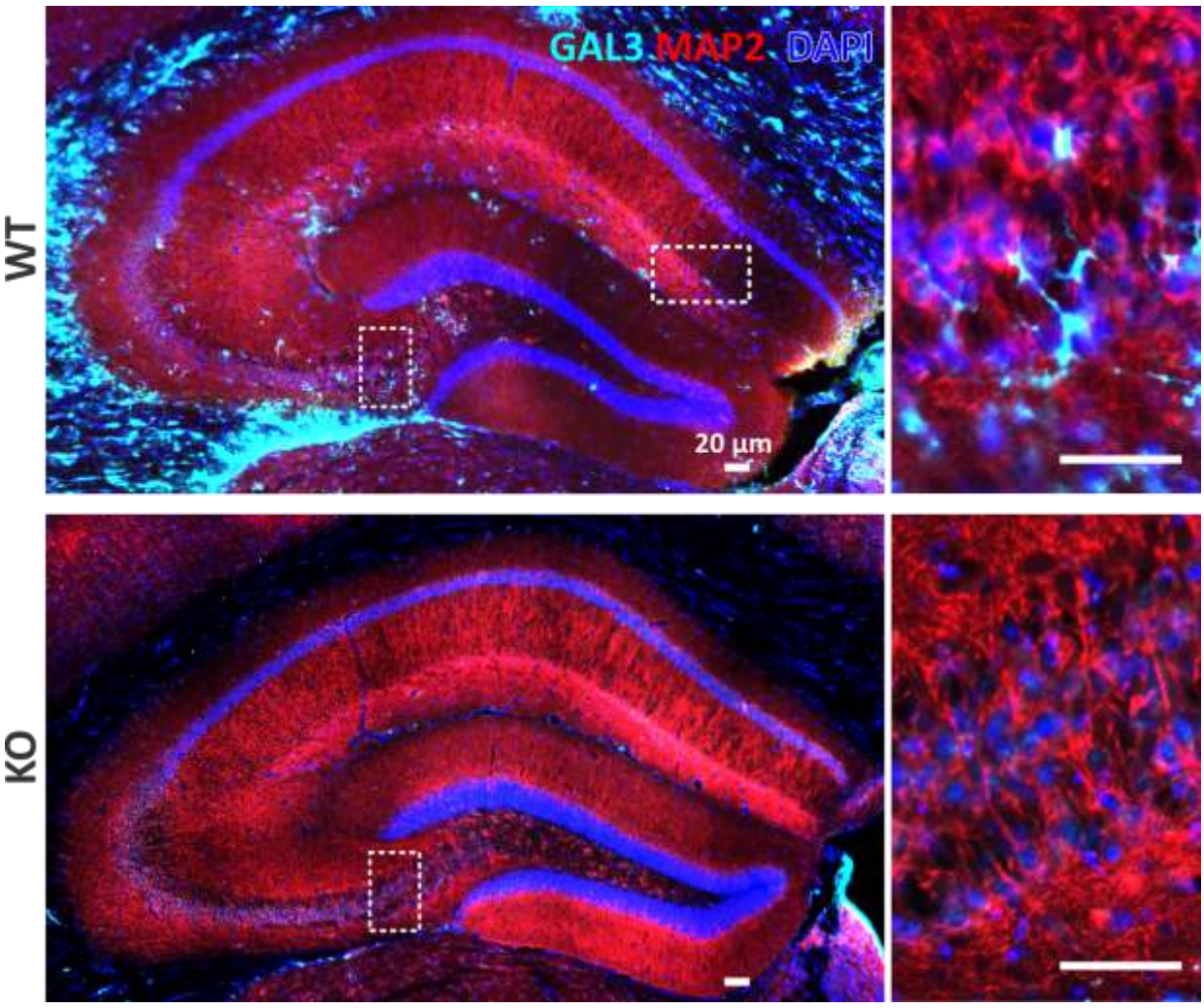
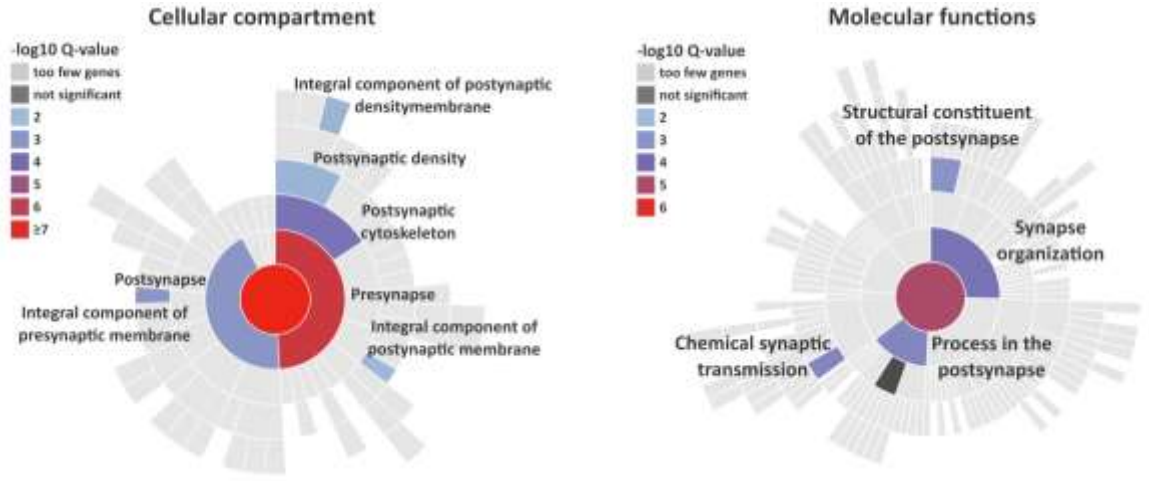


Figure 49. Ndr2 deficiency prevents the expression of Galectin 3 positive cells in the dorsal hippocampus in old mice. 30µm Dorsal hippocampus brain slices taken from 24-month-old Ndr2 WT and KO mice were immunostained against galectin 3 (cyan) and MAP2 (red) (A). n=4.

Ndr2 KO deficiency prevents age-related changes in synaptic pathways in the dorsal hippocampus

As the last step to identify other possible targets that could explain the behavioural phenotype observed in the old KO mice, we subjected the target proteins that are changed during ageing in the WT but not in the KO, and vice versa, to a GO analysis of only synaptic proteins. We used SYNGO, which is a curated database for synapse function and gene enrichment studies freely available online (Figure 50). In the

WT mice, we found 39 synaptic proteins that change during ageing, while in the KO mice, there were none. (Actn1, Anxa5, ApoE, Arhgap44, Atp2b4, Bcas1, Cacng5, Cck, Clu, Dock10, Efnb2, Elavl4, Erbin, Fxyd6, Gabra3, Gap43, Grin3a, Hnrnpa2b1, Hnrnpa3, Ina, Lamp1, Nefl, Plcxd3, Pld1, Plxnc1, Prkcd, Pura, Rpl24, Rpl27a, Rpl35, Rpl7a, Rps9, Snph, Sptbn2, Stau2, Synj2, Synpo, Sypl and Vdac1). The most over-represented GO terms for CC were the synapse, synaptic organization, synaptic transmission and



Cellular component			
Ontology term	Gene count	p-value	q-value
Synapse	34	8,29E-07	1,08E-05
Postsynapse	22	1,48E-05	9,62E-05
Presynapse	18	0,000155	0,000672
Postsynapstic specialization	10	0,00645	0,0012
Postsynaptic density	8	0,0149	0,0242
Postynaptic ribosome	5	0,00228	0,00743
Presynaptic ribosome	4	0,00492	0,0107
Presynaptic active zone	4	0,0276	0,0399
Postsynaptic cytoskeleton	3	0,00343	0,00893
Presynaptic active zone membrane	3	0,0374	0,0486

Biological process			
Ontology term	Gene count	p-value	q-value
Process in the synapse	23	0,0069	0,00332
Synapse organization	9	149	0,0234
Synaptic signaling	6	0,0349	0,0479
Process in the postsynapse	6	0,584	0,0642
Translation in the postsynapse	5	0,00904	0,00332
Chemical synaptic transmission	5	507	0,062
Structural constituent of postsynapse	4	0,00358	0,00332
Translation at presynapse	4	0,0461	0,0127
Postsynaptic modulation of chemical synaptic transmission	3	0,00579	0,0127
Transport	3	0,0012	0,0221

Figure 50. Ndr2 deficiency prevents age-related alterations in synaptic pathways in the dorsal hippocampus. The proteins that changed during ageing in the WT but not the KO and vice versa were analyzed using the open access tool SYNGO. The gene count and enrichment score (q-value) were obtained for the gene ontology terms for Cellular compartment and biological process.

translation. On the other hand, the most represented CC terms were those related to the pre and postsynaptic ribosome and the active zone. Among all the target proteins, it stood out that we found ApoE in the SYNGO analysis and the pathway enrichment analysis. ApoE is a lipoprotein that associates with several kinds of lipid particles like low-density lipoproteins and high-density lipoproteins and transports them intracellularly or across the bloodstream, hence the name Apolipoprotein E. Besides the role as a lipid transporter molecule, in the neurons, it has been shown that ApoE binds to its receptor

activates a transcription cascade involving ERK1/2, and inhibits c-junk signalling in the presence of calcium through the activation of the NMDA receptor[155]. It is thought that by alterations in this mechanism and the lipid transport function, certain alleles of ApoE might play a role in neurodegenerative diseases. One of the most well-studied examples is in AD, as it has been widely demonstrated that among the several alleles of ApoE, some of them highly associate and predict the onset and severity of AD (ApoE computational biology ε3 and ε4). Interestingly, it has been shown that another mechanism of activation is through the activity of the transcription factor C/EBPβ[156]. In mice, this transcription factor increases the transcription of ApoE, whereas in humans, it favours specifically the transcription of the ApoE ε4 allele. It has even been shown that while carriers of the ApoE ε2 allele have improved spatial memory in the star maze, those that carry the ε3 or ε4 show decreased spatial navigation performance[157]. The differential effect of the ε2 allele is even more evident in healthy old individuals, as it has been shown that the carriers of ε2 have increased lifespan than those homo or heterozygous carriers of ε3 and ε4[158]. While in humans there are several alleles of the protein, in mice there is only one. However, the role of the WT ApoE protein in memory particularly during ageing, has not been widely characterized. What has been shown is that ApoE KO mice display an increased risk of atherosclerosis, attributed to a deficiency in lipid metabolism and increased inflammation. As a final possible target to explain the behavioural phenotype of the old KO mice, we decided to confirm the proteomics information by performing an IHCC in DH brain slices of old WT and KO mice (Figure 51). In contrast to what we observed with the Gal 3 staining, old Ndr2 WT and KO mice displayed a more or less homogeneous ApoE staining across the whole hippocampus, independently of the brain region. Nevertheless, we observed neurons that were strongly labelled with ApoE in the subiculum of the CA1 of the WT mice and not in the KO; we also observed positive cell bodies in the DG and CA3, even though

the axons were not so strongly stained. These results confirm that Ndr2 also prevents changes in protein expression and pathways related to translation and transmission in the synapses of the DH.

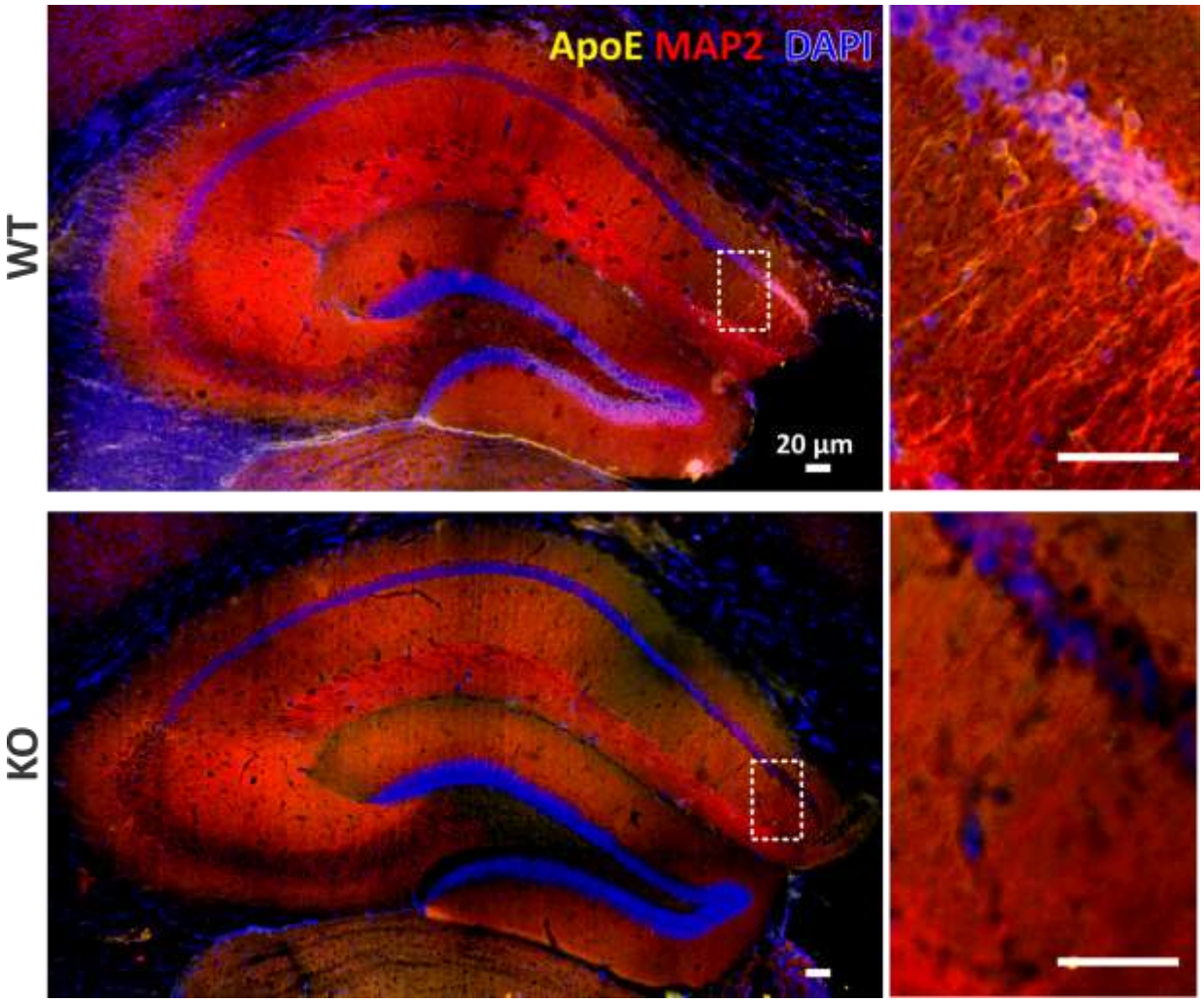


Figure 51. Ndr2 deficiency prevents the expression of ApoE in the dorsal hippocampus in old mice. 30µm Dorsal hippocampus brain slices taken from 24-month-old Ndr2 WT and KO mice were immunostained against ApoE (yellow) and MAP2 (red) (A). n=4.

Discussion

In this final chapter, we analyzed the protein changes that occur in the Old Ndr2 KO mice during ageing, which could provide an explanation and a possible mechanism that explained the increased hippocampal-dependent memory performance and that could be explored deeper in future experiments. First, we observed that an unbiased data analysis showed alterations between WT and KO mice or between old and young mice. These proteins could be grouped into 9 different clusters, each one showing a different effect of age or genotype. When we compared only the changes that occurred in the old vs young mice in each genotype individually, we observed that the changes in the protein expression during ageing in the KO mice were less than those in the WT. Then when we analyzed the pathways that changed during ageing that were not common between the WT and KO mice, we found that the absence of KO prevented changes in pathways related to mitochondrial and ribosomal function, as well as inflammation. One of the most upregulated targets during WT ageing was Galectin 3. We confirmed that the old KO mice show almost no Galectin 3 positive cells in the dorsal hippocampus. Finally, we analyzed the synaptic pathways that change during ageing in the WT mice but not the KO. We found that there were alterations in pathways mainly related to the pre and postsynaptic transmission, pre and postsynaptic translation and in the active zone, and we identified ApoE as a main upstream regulation of synaptic pathways that were altered in the WT mice during ageing. Finally, we confirmed by immunohistochemistry that Ndr2 prevented the upregulation of neuronal ApoE in the dorsal hippocampus.

In conclusion, we showed for the first time, that Ndr2 KO prevents many alterations in the proteome during ageing and that those changes, probably altogether and not a single one individually, can explain why do the old Ndr2 mice have improved hippocampal memory. Even though the autophagy pathway was not altered during ageing, we can´t disregard the importance of the effect in the KO mice. While none of the classic autophagic regulators showed alterations, we observed that Ndr2 KO prevented the upregulation of LAMP1 and Grin3a (NMDA receptor subunit 3A). The former is a lysosomal receptor that targets many signalling molecules to the lysosome. Paradoxically, it has been shown that it is required for autophagy, but also that in some model organisms is dispensable[159]. In any case, it is used as a marker for lysosomal biogenesis, and more Lamp1 usually means more lysosomes or acidic vesicles. This shows that during ageing, there could be an accumulation of lysosomes or endosomes that feed to

the age-related alteration of autophagy and that Ndr2 prevents this effect. For Grin3a, even though the NMDA stimulation induces autophagy, as was described and discussed in the chapters before, there´s no information on whether there is a subunit specificity in the context of autophagy induction. Nonetheless, this possibility can´t be ruled out and deserves further investigation. Besides macroautophagy, the GSEA showed that Ndr2 prevents the upregulation of mitophagy during ageing. Tomm40 and Tomm22 were demonstrated to be necessary for Parkin1-dependent mitophagy, which is the principal type of selective mitophagy in the neurons, and when they are overexpressed, mitophagy is reduced. This mitochondria clearance occurs in healthy conditions; thus, our data would suggest that the maintenance of mitochondrial clearance is prevented during ageing. This would, in turn, increase the damaged mitochondria severely impair energy metabolism and create cellular stress. Unsurprisingly, proper mitophagy is also required for spatial memory. In a mouse model of corticosterone-induced deficiency in mitophagy, it was observed that spatial memory was altered and that pharmacological induction of mitophagy increased the spontaneous alternation performance of the mice[160]. On the pathological side, it is known that mitochondrial dysfunction is a distinctive feature of AD and PD and that it is directly related to the increase in ROS and a decrease in energy metabolites like ATP and NADH that leads to axonal and synaptic dysfunction. Mitochondrial dysfunction is so important to ageing, as previously described, it is considered one of its hallmarks. In that regard, another possibility that has to be seriously considered is that Ndr2 participates in cellular senescence. Icam1, Moesin1, CD44 and even the β-2 Integrin have been implicated in cellular senescence in different types of cells. Nonetheless, probably the best example comes also from Gal3. In one study, it was demonstrated that KD of Gal3 decreased the retinoblastoma protein, Cyclin D1 and also the activity of the transcription factor E2F1[161], all involved in the cell cycle arrest of senescent cells. Moreover, increasing galectin 3 levels in a culture of senescent cells, increased the tumorigenic effect of the media through the upregulation of MAPK and ERK1/2. This is an important piece of evidence because it suggests that besides participating in the cell cycle arrest of senescent cells, it might also be involved in the regulation of the senescence-associated secretory phenotype (SASP). One of the most detrimental and systemic effects observed during ageing is arguably the SASP. In a series of remarkable experiments, senescence was induced either by ionizing radiation, oncogenic transformation or DNA damage in cultured cells that were later transplanted into healthy mice. It was observed in all the cases that the small proportion of grafted cells could induce secondary cellular senescence[162], as well as osteoarthritis[163], suppressed adipogenesis[164], kidney injury[165], and even decreased lifespan[166]. Given the small number of grafted cells, the conclusion is that this effect is achieved solely by the paracrine effect

of the SASP. The effect of grafting senescent cells on brain function has not been assessed yet; nevertheless, it is known that the systemic removal of senescent secretory cells (mostly microglia) in the brain not only reduced the SASP but decreased inflammatory markers in the hippocampus and improved hippocampal-dependent memory[167]. Even though there is still not a clear connection between Ndr2 and Galectin 3, we can draw some conclusions from the literature and our results. It is known that Galectin 3 interacts with integrins in order to regulate cell adhesion and motility. On the other hand, previous work from our lab demonstrated that Ndr2 is activated upstream of the neurite growth dependent on the α1β1 integrin[129], and that Ndr2 KO neurons fail to engage the α1β1 integrin subunit in the context of neurite formation. One hypothesis is that Ndr2 deficiency prevents cell motility and that the absence of the Gal3 positive glial-like cells that we observe in the Old Ndr2 KO mice hippocampus could be because cells failed to migrate into the hippocampus, rather than a reduced intrinsic expression of Gal3.Another possibility that also involves autophagy is that Gal3 participates in the non-canonical secretion of certain molecules through the autophagosome/lysosome pathway, such as α-Synuclein[168]. Interestingly enough, α-Synuclein is also part of the SASP[169]! So, in the KO neurons, it could be possible that alterations in the autophagy pathway lead to less secretion of proinflammatory molecules and less recruitment of Gal3-positive cells that, in turn, reduce inflammation and prevent the cognitive decline associated with ageing. The relationship between cellular senescence and autophagy has been widely discussed, and not a single conclusion can be drawn. There are experiments that both support a positive feedback loop between autophagy impairment and cellular senescence, as well as experiments that show that senescent cells require proper functioning autophagy in order to secrete the SASP[170]. Few molecules and proteins have been found to link the induction of cellular senescence and the SASP with autophagy, such as Gata4; a transcription factor that is stabilized by degradative autophagy and that transcribes many proinflammatory components of the SASP[171]. The possibility that Ndr2 participates in both inflammation, senescence and autophagy with particular interest during ageing is an exciting possibility that needs exploring.

CONCLUSION

In this doctoral thesis, we demonstrated that Ndr2 participates in hippocampal autophagy both in vitro and in vivo. Also, Ndr2 KO mice show defects in basal autophagy both in young and old age in the dorsal hippocampus, and that surprisingly, old mice display better spatial memory than old WT mice. We also demonstrated that pharmacological upregulation of autophagy proved to be detrimental to spatial memory in mice aged more than 24 months old. Finally, we demonstrated that the changes in the dorsal hippocampus go beyond only autophagy. We provide strong evidence that shows that Ndr2 deficiency prevents age-related alterations in inflammation, mitochondrial function, protein translation and synaptic transmission, among others, showing that Ndr2 deficiency prevents age-related changes in proteostasis in the dorsal hippocampus.

In conclusion, this work shows for the first time ever, that autophagy upregulation during ageing might be detrimental for certain tasks and in certain contexts, for instance, in the dorsal hippocampus. Conversely, mice that display defects in autophagy also display a less age-related decline in cognitive capabilities, challenging the current scientific consensus on the effects of autophagy modulation in cognition and ageing. Some mechanisms were discussed in Chapter 2; nonetheless, another interesting observation can be drawn out. The effect of trehalose in the decrease of reactive oxygen species (ROS) has been widely studied. First, it was observed that in response to an enriched trehalose medium, yeast cells upregulate a plethora of antioxidant genes such as thioredoxin reductase and catalase, and this caused an increased rate of growth[172]. Conversely, when yeast cells were grown under increased ROS, trehalose was metabolized in order to cope with the stress. Besides that, it has also been shown that trehalose itself is a scavenger of ROS, particularly of hydrogen peroxide (H_2O_2) and superoxide anions (O_2^-)[173]. This effect translated well into a model of human neuroblastoma cells, in which trehalose prevented autophagic cell death of SH-S5Y5 cells growing under increased H_2O_2. Even though this effect was not completely independent of autophagy upregulation, an off-target beneficial effect was that trehalose decreased endoplasmic reticulum stress, which was believed to be a parallel pro-survival mechanism in this oxidative context[174]. Moreover, there have also been attempts to link trehalose to an improvement in brain function and memory in ageing. A study showed that a transgenic mouse model of D-galactose overexpression that shows increased cellular senescence and reduced exploration in the open field task could be rescued by trehalose administration by systemically increasing the antioxidative upregulation of the liver and brain[175]. All and all, a decrease in ROS and an increase in autophagy would still be considered to be beneficial for brain function, which is still contrary to what we show in this

work. ROS production, particularly during ageing, is often considered to be detrimental and damaging for the cells, particularly neurons that have fewer antioxidant defence mechanisms and that can´t cope well with DNA damage, being post-mitotic cells. Nonetheless, ROS are also required as small signalling molecules, particularly during synaptic plasticity; it has been shown that the upregulation of NMDA receptors, Calcium and Potassium channels, ERK, CREB and CAMKII, among others, follows an increase in ROS production, and even more, that LTP in the CA1 is prevented when O_2^- anions are scavenged with SOD mimicking molecules, and that PKC signalling during LTP requires O_2 as well, showing that ROS production is indeed necessary for plasticity and possibly memory[176–179]. In conclusion, this is also a possible explanation to try to understand the contradictory effect that trehalose has in spatial memory during ageing, and given that trehalose is already commercially available, it has a lot of potential translatability value, so knowing the possible beneficial (or harmful) effects that it could have in humans, whether they´re solely related to the upregulation of autophagy or parallel mechanisms like redox signalling, is of great medical value.

We also characterized even more what was already known, a mouse model that is very well studied in our group, the Ndr2 KO mice. We demonstrated that the known phenotypic behaviours of young mice aged less than 6 months are not necessarily the same in old individuals. we also provided evidence for the first time that Ndr2 might have a strong antagonistic pleiotropic effect, as the evolutionary theory of ageing predicts to be the case for the proteins that are thought to drive the ageing process forward, adding another piece of evidence to the current understanding of ageing. In that regard, it is a fact among many different species that follow similar cycles of life history in which reproduction comes after an early stage of development post-birth (infancy) that any deleterious trait appearing either in infancy or during the reproductive phase that decreases fitness will be strongly unfavored by natural selection, and after a few generations, it will be eliminated from the population. From this observation, it can be deduced that a deleterious trait that appears during infancy will be selected against stronger than if that same deleterious trait that appeared later during the reproductive phase because if such trait so much as prevents reproduction by killing the carrier in childhood it will never pass to the next generation, contrary to traits that appear after reproduction started, but even more, the pressure of natural selection will be even lower if those traits appeared in the later stages of reproduction and null if reproduction has already ceased. In summary, the force of natural selection decreases over lifetime[180]. That´s why any beneficial traits that appear only in later stages of life will not be passed away to the next generation, or in the case of deleterious ones, they will never be eliminated by natural selection, so, over generations, many different deleterious traits that appear later in life will accumulate in the

populations and increase the intrinsic mortality at this stage. From this point of view, we could easily call the period in which the force of natural selection is in decline, ageing. This hypothesis is often referred to as the mutation accumulation theory of ageing, and while it is currently well accepted, it has one major setback, which is that the appearance of either beneficial or deleterious traits later in life is governed by chance, hypothetically, several species that doesn´t age and doesn't show any age-related diseases because only beneficial mutations accumulated in them, could and would exist. That´s why another crucial observation was incorporated into the modern understanding of the evolutionary theory of ageing, which is that if some of the traits that are detrimental during ageing are the consequence of maladaptive or opposite effects of traits that favour fitness in early stages of life, they will be positively selected, and thus increase the pool of deleterious traits during ageing, but the opposite effect (beneficial during ageing, detrimental during infancy), just wouldn't. Traits that follow this pattern are said to have an antagonistic pleiotropic effect, and many, if not all of the nuisances of ageing can be understood under this assumption, for instance, in humans: sustained DNA damage response, oncogenic transformation, loss of proteostasis, cellular senescence or age-related diseases such as cystic fibrosis, Huntington's disease, osteoporosis, Alzheimer's disease, etc. As was discussed in previous chapters, Ndr2 is involved in many important processes like plasticity, migration, regulation of autophagy, cell cycle control, etc. Nonetheless, we also showed evidence that the absence of Ndr2 reduces inflammation, alterations in translation, etc., during ageing. We could hypothesize that Ndr2 helps immune cells migrate and combat infectious diseases or reorganize the ECM in the context of plasticity during young age, but also that it reinforces the increased migration and accumulation of immune cells that produce inflammation during ageing. Another example of its pleiotropic effect could be that in young mice, Ndr2 regulates the early steps of autophagy, which in turn is necessary for correct synaptic transmission, clearance of protein aggregates and remodelling of the synapses. Still, during ageing, when the robustness of autophagy regulation is lost, Ndr2 maladaptively contributes to synaptic degeneration and the aberrant degradation of cargo by autophagy that assists in the loss of cognition, another speculation that could be taken out from what we know and was presented about Ndr2 in this work, relates to its role in dendrites; At the same time, Ndr2 is required for correct dendritic branching and arborization needed for correct spine formation in young age, but during ageing, it could hinder synaptic pruning and reduce plasticity. A final possibility worth mentioning, and that was hinted at in the last chapter, relates to its role in cell cycle regulation. It is known that Ndr2 regulates cell cycle progression by direct phosphorylation of p21, which results in the retention of p21 in the cytoplasm, and that when Ndr2 is knocked down, this causes cell cycle arrest through p21[181]. This effect could help maintain the stemness of cells during development and at a young age and help the malignant

transformation and tumorigenesis during ageing; nevertheless, a more interesting possibility that can explain the phenotypic behaviour relates to the fact that it has been observed in several parallel experiments and different model organisms, that neurons can actually re-enter the cell cycle and that even though this can have beneficial effects such as the repair of DNA damage, it also causes a plethora of alterations[182], that are mostly related to neurodegeneration in AD-like, resistance to B amyloid induced neuronal death[183], increased Tau stability and synaptic loss[184]. This observation has acquired a lot of attention, to the extent that the cell-cycle re-entry in AD is being seriously investigated as the actual cause of the disease. Some models predict that p21 participates in this phenomenon, so it could easily be hypothesized that during ageing, the regulation of p21 by Ndr2 suffers alterations that lead to this aberrant cell cycle re-entry and, as a consequence, alterations in neuronal and brain function.

Finally, the role of ApoE in the modulation of cognition is worth mentioning. Interestingly enough, not much is known about the role of the wild-type ApoE in mice, in harsh contrast to its human counterpart, where a very allele-specific effect has been widely characterized. From the evidence in humans, it is clear that ApoE plays a role in cognition and memory during ageing; it is widely accepted that the ApoE ε4 allele is one of the main risk factors for developing neurodegenerative diseases like AD and PD, whereas the ε2 allele has been shown to reduce the risk of dementia and cognitive impairment. Nonetheless, the role of ApoE in memory seems to be less straightforward than it seems. A very interesting observation is that a positive correlation between de ApoE ε4 gene dosage and a decreased risk of cognitive impairment in healthy middle-aged men was observed, but not in females, showing that even though the carriers have an increased risk of developing AD, in non-pathological conditions, ApoE ε4 positively regulates short term memory[185]. The opposite was observed in middle-aged and old patients with mild cognitive impairment, where ε4 carriers had decreased performance in subjective organization[186], which is a higher cognitive function that involves the cingulate cortex, temporal lobe, and hippocampus, among others[187,188]. Another parallel study also demonstrated that while healthy adult ApoE ε4 carriers display long-term object location memory deficits, they also show increased short-term memory performance in a virtual memory array paradigm[189]. A final piece of evidence that demonstrates a role of ApoE in neuronal function not related to disease is that ApoE is required for dendritic arborization in hippocampal neurons, very much like Ndr2, and hippocampal development and neurogenesis[190]. An interesting possibility that can be taken from these observations is that there might be an interaction between Ndr2 and ApoE and that during ageing, Ndr2 participates in the maladaptive roles of ApoE in memory loss and in pathogenesis, providing another parallel explanation of how Ndr2 deficiency could prevent memory impairment. Additionally, these published works strongly

suggest that ApoE has antagonistic pleiotropic functions related to memory, such as could be the case for Ndr2, suggesting a fundamental role in brain development in different stages and cognition during ageing.

In a brief summary, when Ndr2 is viewed as an ageing factor with antagonistic and pleiotropic traits ranging from autophagy to cell cycle control and neurite growth, it can easily be understood how during ageing, it could contribute to a decrease in cognition and neuronal function. Taking everything into account, this work also opens the possibility of exploring the role of Ndr2 in processes that are extremely relevant for ageing research, such as mitophagy, cellular senescence, and even neurodegeneration.

I want to conclude by saying that the work I did during my PhD created the theoretical and experimental framework that highlights Ndr2 as a novel ageing factor with antagonistic pleiotropic traits. Unexpectedly, I discovered that this kinase might lie comfortably at the crossroads of many converging mechanisms contributing to the loss of cognition during ageing. Nonetheless, there is a lot more to understand about Ndr2, so it can help us understand, in turn, a little bit more about what is ageing, why we age, and what lies ahead.

LIST OF ABBREVIATIONS

AD	Alzheimmer´s disease
ALS	Amyotrophic lateral sclerosis
AP	Autophagosomes
CA1	Cornu ammonis 1
CA3	Cornu ammonis 3
CMA	Chaperone mediated autophagy
CQ	Chloroquine
Ctrl	Control
DDR	DNA damage response
DG	Dentate gytus
DH	Dorsal hippocampus
DI	Discrimination index
DIV	Days *in vitro*
ER	Endoplasmic reticulum
GO	Gene ontology
HD	Huntington´s disease
ICC	Immonocytochemistry
IHCC	Immunohistochemistry
KO	Knock Out
LC3	Light chain 3
LTD	Long term depression
LTP	Long term potentiation
MWM	Morris water maze
Ndr2	Nuclear Dbf2-related protein
NE	Northeast
NMDA	N-methyl-D-aspartate
NOL	Novel object location
NOR	Novel object recognition
OF	Open field
PD	Parkinson´s disease
RM	Repeated meassurements
ROS	Reactive oxygen species
RR	Rotarod
SASP	Senescence associated secretory phenotype
Sirt1	Sirtuin 1
Sp. Alt.	Spontaneous alternation
SW	Southwest
TFEB	Transcription factor EB
Treh	Trehalose
WT	Wild Type

REFERENCES

1. WHO. World report on Ageing & Health. (2015).
2. Hindle, A. G., Lawler, J. M., Campbell, K. L. & Horning, M. Muscle senescence in short-lived wild mammals, the soricine shrews blarina brevicauda and sorex palustris. *J Exp Zool A Ecol Genet Physiol* **311**, 358–367 (2009).
3. Ruby, J. G., Smith, M. & Buffenstein, R. Naked mole-rat mortality rates defy Gompertzian laws by not increasing with age. *Elife* **7**, (2018).
4. Sherman, P. W. & Jarvis, J. U. M. Extraordinary life spans of naked mole-rats (Heterocephalus glaber). *J Zool* **258**, 307–311 (2002).
5. Kirkwood, T. B. L. The origins of human ageing. *Philosophical Transactions of the Royal Society B: Biological Sciences* **352**, 1765–1772 (1997).
6. Austad, N. Retarded senescence in an insular population of Virginia opossums. *J. Zool.* **229**, 695–708 (1993).
7. López-Otín, C., Blasco, M. A., Partridge, L., Serrano, M. & Kroemer, G. The hallmarks of aging. *Cell* **153**, (2013).
8. Lu, M. H. *et al.* Intranasal Transplantation of Human Neural Stem Cells Ameliorates Alzheimer's Disease-Like Pathology in a Mouse Model. *Front Aging Neurosci* **13**, (2021).
9. Shields, H. J., Traa, A. & van Raamsdonk, J. M. Beneficial and Detrimental Effects of Reactive Oxygen Species on Lifespan: A Comprehensive Review of Comparative and Experimental Studies. *Frontiers in Cell and Developmental Biology* vol. 9 Preprint at https://doi.org/10.3389/fcell.2021.628157 (2021).
10. Kandlur, A., Satyamoorthy, K. & Gangadharan, G. Oxidative Stress in Cognitive and Epigenetic Aging: A Retrospective Glance. *Frontiers in Molecular Neuroscience* vol. 13 Preprint at https://doi.org/10.3389/fnmol.2020.00041 (2020).
11. Irvine, G. B., El-Agnaf, O. M., Shankar, G. M. & Walsh, D. M. Protein aggregation in the brain: The molecular basis for Alzheimer's and Parkinson's diseases. *Molecular Medicine* vol. 14 451–464 Preprint at https://doi.org/10.2119/2007-00100.Irvine (2008).
12. Glagtigny, M., Moriceau, S., Rivagorda, ManonMorel, E., Codogono, P. & Oury, F. Autophagy is Required for Memory Formation and Reverses Age-Related Memory Decline. *Current Biology* **29**, 435–448 (2019).
13. Lee, C.-W. *et al.* Selective autophagy degrades nuclear pore complexes. *Nat Cell Biol* **22**, 159–166 (2020).
14. Aman, Y. *et al.* Autophagy in healthy aging and disease. *Nature Aging* vol. 1 634–650 Preprint at https://doi.org/10.1038/s43587-021-00098-4 (2021).
15. Li, W. W., Li, J. & Bao, J. K. Microautophagy: Lesser-known self-eating. *Cellular and Molecular Life Sciences* **69**, 1125–1136 (2012).
16. Oku, M. & Sakai, Y. Three Distinct Types of Microautophagy Based on Membrane Dynamics and Molecular Machineries. *BioEssays* **40**, 1–6 (2018).
17. Kaushik, S. *et al.* Chaperone-mediated autophagy at a glance. *J Cell Sci* **124**, 495–499 (2011).
18. Kaur, J. & Debnath, J. Autophagy at the crossroads of catabolism and anabolism. *Nat Rev Mol Cell Biol* **16**, 461–472 (2015).

19. Takeshige, K., Baba, M., Tsuboi, S., Noda, T. & Ohsumi, Y. Autophagy in yeast demonstrated with proteinase-deficient mutants and conditions for its induction. *Journal of Cell Biology* **119**, 301–312 (1992).
20. Rabinowitz, J. D. & White, E. Autophagy and metabolism. *Science (1979)* **330**, 1344–1348 (2010).
21. Onodera, J. & Ohsumi, Y. Autophagy is required for maintenance of amino acid levels and protein synthesis under nitrogen starvation. *Journal of Biological Chemistry* **280**, 31582–31586 (2005).
22. Madrigal-Matute, J. & Cuervo, A. M. Regulation of Liver Metabolism by Autophagy. *Gastroenterology* vol. 150 328–339 Preprint at https://doi.org/10.1053/j.gastro.2015.09.042 (2016).
23. Valencia, M., Kim, S. R., Jang, Y. & Lee, S. H. Neuronal autophagy: Characteristic features and roles in neuronal pathophysiology. *Biomol Ther (Seoul)* **29**, 605–614 (2021).
24. Eliopoulos, A. G., Havaki, S. & Gorgoulis, V. G. DNA damage response and autophagy: A meaningful partnership. *Front Genet* **7**, 1–13 (2016).
25. Frankel, L. B., Lubas, M. & Lund, A. H. Emerging connections between RNA and autophagy. *Autophagy* **13**, 3–23 (2017).
26. Joy, S. *et al.* Basal and starvation-induced autophagy mediates parasite survival during intraerythrocytic stages of Plasmodium falciparum. *Cell Death Discov* **4**, (2018).
27. Kuma, A. *et al.* The role of autophagy during the early neonatal starvation period. *Nature* **432**, 1032–1036 (2004).
28. Kang, C., You, N. J. & Avery, L. Dual roles of autophagy in the survival of Caenorhabditis elegans during starvation. *Genes Dev* **21**, 2161–2171 (2007).
29. Turco, E., Fracchiolla, D. & Martens, S. Recruitment and Activation of the ULK1/Atg1 Kinase Complex in Selective Autophagy. *J Mol Biol* 123–134 (2019) doi:10.1016/j.jmb.2019.07.027.
30. Zachari, M. & Ganley, I. G. The mammalian ULK1 complex and autophagy initiation. *Essays Biochem* **61**, 585–596 (2017).
31. Kim, J., Kundu, M., Viollet, B. & Guan, K. L. AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. *Nat Cell Biol* **13**, 132–141 (2011).
32. Takahashi, Y., Young, M. M. & Wang, H. G. SNAPping off Golgi membranes for autophagosome formation. *Cell Cycle* **12**, 15–16 (2013).
33. Hailey, D. W. *et al.* Mitochondria supply membranes for autophagosome biogenesis during starvation. *Cell* **141**, 656–667 (2011).
34. Wei, Y., Liu, M., Li, X., Liu, J. & Li, H. Origin of the Autophagosome Membrane in Mammals. *Biomed Res Int* **2018**, 1012789 (2018).
35. Hagen, C. *et al.* Structural Basis of Vesicle Formation at the Inner Nuclear Membrane. *Cell* **163**, 1692–1701 (2015).
36. Murrow, L. & Debnath, J. A nuclear option that initiates autophagy. *Mol Cell* **57**, 393–395 (2015).
37. Menon, M. B. & Dhamija, S. Beclin 1 phosphorylation - at the center of autophagy regulation. *Front Cell Dev Biol* **6**, 1–9 (2018).
38. Park, J. M. *et al.* ULK1 phosphorylates Ser30 of BECN1 in association with ATG14 to stimulate autophagy induction. *Autophagy* **14**, 584–597 (2018).
39. Russell, R. C. *et al.* HHS Public Access. **15**, 741–750 (2014).
40. Schaaf, M. B. E., Keulers, T. G., Vooijs, M. A. & Rouschop, K. M. A. LC3/GABARAP family proteins: Autophagy-(un)related functions. *FASEB Journal* **30**, 3961–3978 (2016).

41. Maruyama, T. & Noda, N. N. Autophagy-regulating protease Atg4: Structure, function, regulation and inhibition. *Journal of Antibiotics* **71**, 72–78 (2018).
42. Nath, S. *et al.* Lipidation of the LC3/GABARAP family of autophagy proteins relies on a membrane-curvature-sensing domain in Atg3. *Nat Cell Biol* **16**, 415–424 (2014).
43. Birgisdottir, Å. B., Lamark, T. & Johansen, T. The LIR motif - crucial for selective autophagy. *J Cell Sci* **126**, 3237–3247 (2013).
44. Isakson, P., Holland, P. & Simonsen, A. The role of ALFY in selective autophagy. *Cell Death and Differentiation* vol. 20 12–20 Preprint at https://doi.org/10.1038/cdd.2012.66 (2013).
45. Cebollero, E., Reggiori, F. & Kraft, C. Reticulophagy and ribophagy: Regulated degradation of protein production factories. *Int J Cell Biol* **2012**, (2012).
46. Nakatogawa, H. & Mochida, K. Reticulophagy and nucleophagy: New findings and unsolved issues. *Autophagy* **11**, 2377–2378 (2015).
47. Akinduro, O. *et al.* Constitutive Autophagy and Nucleophagy during Epidermal Differentiation. *Journal of Investigative Dermatology* **136**, 1460–1470 (2016).
48. Deng, Z. *et al.* Autophagy Receptors and Neurodegenerative Diseases. *Trends Cell Biol* **27**, 491–504 (2017).
49. Taguchi-Atarashi, N. *et al.* Modulation of local Ptdins3P levels by the PI phosphatase MTMR3 regulates constitutive autophagy. *Traffic* **11**, 468–478 (2010).
50. Nakamura, S. & Yoshimori, T. New insights into autophagosome-lysosome fusion. *J Cell Sci* **130**, 1209–1216 (2017).
51. Maday, S., Wallace, K. E. & Holzbaur, E. L. F. Autophagosomes initiate distally and mature during transport toward the cell soma in primary neurons. *Journal of Cell Biology* **196**, 407–417 (2012).
52. Maday, S. Mechanisms of neuronal homeostasis: Autophagy in the axon. *Brain Res* **1649**, 143–150 (2016).
53. Murdoch, J. D. *et al.* Endophilin-A Deficiency Induces the Foxo3a-Fbxo32 Network in the Brain and Causes Dysregulation of Autophagy and the Ubiquitin-Proteasome System. *Cell Rep* **17**, 1071–1086 (2016).
54. Soukup, S. F. *et al.* A LRRK2-Dependent EndophilinA Phosphoswitch Is Critical for Macroautophagy at Presynaptic Terminals. *Neuron* **92**, 829–844 (2016).
55. Hernandez, D. *et al.* Regulation of Presynaptic Neurotransmission by Macroautophagy. *Neuron* **74**, 277–284 (2012).
56. Kononenko, N. L. *et al.* Retrograde transport of TrkB-containing autophagosomes via the adaptor AP-2 mediates neuronal complexity and prevents neurodegeneration. *Nat Commun* **8**, (2017).
57. Lee, J.-A. Neuronal Autophagy: A Housekeeper or a Fighter in Neuronal Cell Survival? *Exp Neurobiol* **21**, 1–8 (2012).
58. Stavoe, A. K. H., Hill, S. E., Hall, D. H. & Colón-Ramos, D. A. KIF1A/UNC-104 Transports ATG-9 to Regulate Neurodevelopment and Autophagy at Synapses. *Dev Cell* **38**, 171–185 (2016).
59. Kulkarni, A., Chen, J. & Maday, S. Neuronal autophagy and intercellular regulation of homeostasis in the brain. *Curr Opin Neurobiol* **51**, 29–36 (2018).
60. Nikoletopoulou, V., Sidiropoulou, K., Kallergi, E., Dalezios, Y. & Tavernarakis, N. Modulation of Autophagy by BDNF Underlies Synaptic Plasticity. *Cell Metab* **26**, 230-242.e5 (2017).
61. Kaushik, S. *et al.* Autophagy in hypothalamic agrp neurons regulates food intake and energy balance. *Cell Metab* **14**, 173–183 (2011).

62. Caloric restriction stimulates autophagy in rat cortical neurons through neuropeptide Y and ghrelin receptors activation.
63. Aveleira, C. A. *et al.* Neuropeptide Y stimulates autophagy in hypothalamic neurons. *Proc Natl Acad Sci U S A* **112**, E1642–E1651 (2015).
64. Shen, H., Zhu, H., Panja, D., Gu, Q. & Li, Z. Autophagy controls the induction and developmental decline of NMDAR-LTD through endocytic recycling. *Nat Commun* **11**, (2020).
65. Compans, B. *et al.* NMDAR-dependent long-term depression is associated with increased short term plasticity through autophagy mediated loss of PSD-95. *Nat Commun* **12**, (2021).
66. Kallergi, E. *et al.* Dendritic autophagy degrades postsynaptic proteins and is required for long-term synaptic depression in mice. *Nat Commun* **13**, (2022).
67. Liu, J. & Li, L. Targeting Autophagy for the Treatment of Alzheimer's Disease: Challenges and Opportunities. *Front Mol Neurosci* **12**, 1–9 (2019).
68. Cataldo, A. M. *et al.* Presenilin Mutations in Familial Alzheimer Disease and Transgenic Mouse Models Accelerate Neuronal Lysosomal Pathology. *J Neuropathol Exp Neurol* **63**, 821–830 (2004).
69. Caballero, B. *et al.* Interplay of pathogenic forms of human tau with different autophagic pathways. *Aging Cell* **17**, 1–17 (2018).
70. Klucken, J. *et al.* Alpha-synuclein aggregation involves a bafilomycin A1-sensitive autophagy pathway. *Autophagy* **8**, 754–766 (2012).
71. Chartier-Harlin, M. C. *et al.* α-synuclein locus duplication as a cause of familial Parkinson's disease. *Lancet* **364**, 1167–1169 (2004).
72. Tanaka, A. Parkin-mediated selective mitochondrial autophagy, mitophagy: Parkin purges damaged organelles from the vital mitochondrial network. *FEBS Lett* **584**, 1386–1392 (2010).
73. Ochaba, J. *et al.* Potential function for the Huntingtin protein as a scaffold for selective autophagy. *Proc Natl Acad Sci U S A* **111**, 16889–16894 (2014).
74. Croce, K. R. & Yamamoto, A. A role for autophagy in Huntington's disease. *HHS Public Acces* **122**, 16–22 (2019).
75. Valenzuela, V., Nassif, M. & Hetz, C. Unraveling the role of motoneuron autophagy in ALS. *Autophagy* **14**, 733–737 (2018).
76. Hetz, C. *et al.* XBP-1 deficiency in the nervous system protects against amyotrophic lateral sclerosis by increasing autophagy. *Genes Dev* **23**, 2294–2306 (2009).
77. Rudnick, N. D. *et al.* Distinct roles for motor neuron autophagy early and late in the SOD1G93A mouse model of ALS. *Proc Natl Acad Sci U S A* **114**, E8294–E8303 (2017).
78. Caramés, B., Taniguchi, N., Otsuki, S., Blanco, F. J. & Lotz, M. Autophagy is a protective mechanism in normal cartilage, and its aging-related loss is linked with cell death and osteoarthritis. *Arthritis Rheum* **62**, 791–801 (2010).
79. Yang, X. *et al.* Autophagy and Age-Related Eye Diseases. *Biomed Res Int* **2019**, (2019).
80. Abdellatif, M., Sedej, S., Carmona-Gutierrez, D., Madeo, F. & Kroemer, G. Autophagy in cardiovascular aging. *Circ Res* **123**, 803–824 (2018).
81. Quan, W., Lim, Y. M. & Lee, M. S. Role of autophagy in diabetes and endoplasmic reticulum stress of pancreatic β-cells. *Exp Mol Med* **44**, 81–88 (2012).
82. Raz, N. *et al.* Regional brain changes in aging healthy adults: General trends, individual differences and modifiers. *Cerebral Cortex* **15**, 1676–1689 (2005).

83. Moffat, S. D., Zonderman, A. B. & Resnick, S. M. Age differences in spatial memory in a virtual environment navigation task. *Neurobiol Aging* **22**, 787–796 (2001).
84. Jansen, P., Schmelter, A. & Heil, M. Spatial knowledge acquisition in younger and elderly adults: A study in a virtual environment. *Exp Psychol* **57**, 54–60 (2009).
85. Klencklen, G., Després, O. & Dufour, A. What do we know about aging and spatial cognition? Reviews and perspectives. *Ageing Res Rev* **11**, 123–135 (2012).
86. Bird, C. M. & Burgess, N. The hippocampus and memory: Insights from spatial processing. *Nat Rev Neurosci* **9**, 182–194 (2008).
87. Zhu, H. *et al.* Impairments of spatial memory in an Alzheimer's disease model via degeneration of hippocampal cholinergic synapses. *Nat Commun* **8**, (2017).
88. Xu, B. L. *et al.* Effects of Caloric Intake on Learning and Memory Function in Juvenile C57BL/6J Mice. *Biomed Res Int* **2015**, (2015).
89. Kuhla, A. *et al.* Lifelong Caloric Restriction Increases Working Memory in Mice. *PLoS One* **8**, (2013).
90. Emanuele, E. *et al.* Can enhanced autophagy be associated with human longevity? Serum levels of the autophagy biomarker beclin-1 are increased in healthy centenarians. *Rejuvenation Res* **17**, 518–524 (2014).
91. Wang, X. F. *et al.* Transcriptome evidence reveals enhanced autophagy-lysosomal function in centenarians. *Genome Res* **28**, 1601–1610 (2018).
92. Mattison, J. A. *et al.* Caloric restriction improves health and survival of rhesus monkeys. *Nat Commun* **8**, (2017).
93. Willis, C. M. *et al.* Astrocyte Support for Oligodendrocyte Differentiation can be Conveyed via Extracellular Vesicles but Diminishes with Age. *Sci Rep* **10**, 1–14 (2020).
94. Chávez, M. N. *et al.* Autophagy Activation in Zebrafish Heart Regeneration. *Sci Rep* **10**, 2191 (2020).
95. Harrison, D. E. *et al.* Rapamycin fed late in life extends lifespan in genetically heterogeneous mice. *Nature* **460**, 392–395 (2009).
96. Orenstein, S. J. *et al.* Interplay of LRRK2 with chaperone-mediated autophagy. *Nat Neurosci* **16**, 394–406 (2013).
97. Rodriguez-Navarro, J. A. *et al.* Inhibitory effect of dietary lipids on chaperone-mediated autophagy. *Proc Natl Acad Sci U S A* **109**, (2012).
98. Zhang, C. & Cuervo, A. M. Restoration of chaperone-mediated autophagy in aging liver improves cellular maintenance and hepatic function. *Nat Med* **14**, 959–965 (2008).
99. Kaushik, S. & Cuervo, A. M. *The coming of age of chaperone-mediated autophagy*. vol. 19 (2019).
100. Krüger, U., Wang, Y., Kumar, S. & Mandelkow, E. M. Autophagic degradation of tau in primary neurons and its enhancement by trehalose. *Neurobiol Aging* **33**, 2291–2305 (2012).
101. Rusmini, P. *et al.* Trehalose induces autophagy via lysosomal-mediated TFEB activation in models of motoneuron degeneration. *Autophagy* **15**, 631–651 (2019).
102. Hergovich, A., Stegert, M. R., Schmitz, D. & Hemmings, B. A. NDR kinases regulate essential cell processes from yeast to humans. *Nat Rev Mol Cell Biol* **7**, 253–264 (2006).
103. Hergovich, A. The roles of NDR protein kinases in hippo signalling. *Genes (Basel)* **7**, 1–16 (2016).
104. Rehberg, K. *et al.* The serine/threonine kinase Ndr2 controls integrin trafficking and integrin-dependent neurite growth. *Journal of Neuroscience* **34**, 5342–5354 (2014).
105. Demiray, Y. E., Rehberg, K., Kliche, S. & Stork, O. Ndr2 Kinase Controls Neurite Outgrowth and Dendritic Branching Through α1 Integrin Expression. *Front Mol Neurosci* **11**, 1–11 (2018).

106. Keller, M. *et al.* NDR2 kinase contributes to cell invasion and cytokinesis defects induced by the inactivation of RASSF1A tumor-suppressor gene in lung cancer cells. *Journal of Experimental and Clinical Cancer Research* **38**, 1–16 (2019).
107. Stegert, M. R., Tamaskovic, R., Bichsel, S. J., Hergovich, A. & Hemmings, B. A. Regulation of NDR2 protein kinase by multi-site phosphorylation and the S100B calcium-binding protein. *Journal of Biological Chemistry* **279**, 23806–23812 (2004).
108. Bichsel, S. J., Tamaskovic, R., Stegert, M. R. & Hemmings, B. A. Mechanism of activation of NDR (nuclear Dbf2-related) protein kinase by the hMOB1 protein. *Journal of Biological Chemistry* **279**, 35228–35235 (2004).
109. Cornils, H., Kohler, R. S., Hergovich, A. & Hemmings, B. A. Downstream of human NDR kinases: Impacting on c-myc and p21 protein stability to control cell cycle progression. *Cell Cycle* **10**, 1897–1904 (2011).
110. Léger, H. *et al.* Ndr kinases regulate retinal interneuron proliferation and homeostasis. *Sci Rep* **8**, 1–21 (2018).
111. Schmitz-Rohmer, D. *et al.* NDR kinases are essential for somitogenesis and cardiac looping during mouse embryonic development. *PLoS One* **10**, (2015).
112. Du, Z., Tong, X. & Ye, X. Cyclin D1 promotes cell cycle progression through enhancing NDR1/2 kinase activity independent of cyclin-dependent kinase. *Journal of Biological Chemistry* **288**, 26678–26687 (2013).
113. Tang, Y. & Yu, W. SIRT1 and p300/CBP regulate the reversible acetylation of serine-threonine kinase NDR2. *Biochem Biophys Res Commun* **518**, 396–401 (2019).
114. Zu, Y. *et al.* SIRT1 promotes proliferation and prevents senescence through targeting LKB1 in primary porcine aortic endothelial cells. *Circ Res* **106**, 1384–1393 (2010).
115. Xu, Z. *et al.* The miR-29b-Sirt1 axis regulates self-renewal of mouse embryonic stem cells in response to reactive oxygen species. *Cell Signal* **26**, 1500–1505 (2014).
116. Lee, S. H., Lee, J. H., Lee, H. Y. & Min, K. J. Sirtuin signaling in cellular senescence and aging. *BMB Rep* **52**, 24–34 (2019).
117. Satoh, A. *et al.* Sirt1 extends life span and delays aging in mice through the regulation of Nk2 Homeobox 1 in the DMH and LH. *Cell Metab* **18**, 416–430 (2013).
118. Kilic, U. *et al.* A remarkable age-related increase in SIRT1 protein expression against oxidative stress in elderly: SIRT1 gene variants and longevity in human. *PLoS One* **10**, 1–19 (2015).
119. Mao, K. *et al.* Late-life targeting of the IGF-1 receptor improves healthspan and lifespan in female mice. *Nat Commun* **9**, 1–12 (2018).
120. Stallone, G., Infante, B., Prisciandaro, C. & Grandaliano, G. MTOR and aging: An old fashioned dress. *Int J Mol Sci* **20**, 1–17 (2019).
121. Hansen, M., Rubinsztein, D. C. & Walker, D. W. Autophagy as a promoter of longevity: insights from model organisms. *Nat Rev Mol Cell Biol* **19**, 579–593 (2018).
122. Emoto, K. *et al.* Control of dendritic branching and tiling by the tricornered-kinase/furry signaling pathway in Drosophila sensory neurons. *Cell* **119**, 245–256 (2004).
123. Natarajan, R. *et al.* Tricornered kinase regulates synapse development by regulating the levels of Wiskott-Aldrich syndrome protein. *PLoS One* **10**, 1–21 (2015).

124. Zallen, J. A., Peckol, E. L., Tobin, D. M. & Bargmann, C. I. Neuronal cell shape and neurite initiation are regulated by the Ndr kinase SAX-1, a member of the Orb6/COT-1/warts serine/threonine kinase family. *Mol Biol Cell* **11**, 3177–3190 (2000).
125. Yang, R., Kong, E., Jin, J., Hergovich, A. & Püschel, A. W. Rassf5 and Ndr kinases regulate neuronal polarity through Par3 phosphorylation in a novel pathway. *J Cell Sci* **127**, 3463–3476 (2014).
126. Critchley, D. R. Cytoskeletal proteins talin and vinculin in integrin-mediated adhesion. *Biochem Soc Trans* **32**, 831–836 (2004).
127. Shi, Y. & Ethell, I. M. Integrins control dendritic spine plasticity in hippocampal neurons through NMDA receptor and Ca2+/calmodulin-dependent protein kinase II-mediated actin reorganization. *Journal of Neuroscience* **26**, 1813–1822 (2006).
128. Kilinc, D. The emerging role of mechanics in synapse formation and plasticity. *Front Cell Neurosci* **12**, 1–9 (2018).
129. Chan, C. S. *et al.* α3-Integrins are required for hippocampal long-term potentiation and working memory. *Learning and Memory* **14**, 606–615 (2007).
130. Wiera, G. *et al.* Integrins bidirectionally regulate the efficacy of inhibitory synaptic transmission and control GABAergic plasticity. *The Journal of Neuroscience* JN-RM-1458-21 (2022) doi:10.1523/jneurosci.1458-21.2022.
131. Chan, C. S., Weeber, E. J., Kurup, S., Sweatt, J. D. & Davis, R. L. Integrin requirement for hippocampal synaptic plasticity and spatial memory. *Journal of Neuroscience* **23**, 7107–7116 (2003).
132. Chan, C. S. *et al.* β1-integrins are required for hippocampal AMPA receptor-dependent synaptic transmission, synaptic plasticity, and working memory. *Journal of Neuroscience* **26**, 223–232 (2006).
133. Lilja, J. & Ivaska, J. Integrin activity in neuronal connectivity. *J Cell Sci* **131**, 1–11 (2018).
134. Joffre, C. *et al.* The pro-apoptotic STK38 kinase is a new Beclin1 Partner positively regulating autophagy. *Current Biology* **25**, 2479–2492 (2015).
135. Shehata, M., Matsumura, H., Okubo-Suzuki, R., Ohkawa, N. & Inokuchi, K. Neuronal stimulation induces autophagy in hippocampal neurons that is involved in AMPA receptor degradation after chemical long-term depression. *Journal of Neuroscience* **32**, 10413–10422 (2012).
136. Koganezawa, N., Hanamura, K., Sekino, Y. & Shirao, T. The role of drebrin in dendritic spines. *Molecular and Cellular Neuroscience* **84**, 85–92 (2017).
137. Gonzalez, C. D., Resnik, R. & Vaccaro, M. I. Secretory Autophagy and Its Relevance in Metabolic and Degenerative Disease. *Front Endocrinol (Lausanne)* **11**, 1–12 (2020).
138. Shin, J. *et al.* Four-week individual caging of male ICR mice alters body composition without change in body mass. *Sci Rep* **8**, 1–6 (2018).
139. Puertollano, R., Ferguson, S. M., Brugarolas, J. & Ballabio, A. The complex relationship between TFEB transcription factor phosphorylation and subcellular localization . *EMBO J* **37**, 1–12 (2018).
140. Sha, Y., Rao, L., Settembre, C., Ballabio, A. & Eissa, N. T. STUB 1 regulates TFEB -induced autophagy–lysosome pathway . *EMBO J* **36**, 2544–2552 (2017).
141. García-Rodríguez, D. & Giménez-Cassina, A. Ketone Bodies in the Brain Beyond Fuel Metabolism: From Excitability to Gene Expression and Cell Signaling. *Front Mol Neurosci* **14**, 1–14 (2021).
142. Wimmer, M., Hernandez, P., Blackwell, J. & Abel, T. Aging impairs hippocampus-dependent long-term memory for object location in mice. *Journal of Investigative Dermatology* **33**, 2220–2224 (2011).
143. Gazova, I. *et al.* Spatial navigation in young versus older adults. *Front Aging Neurosci* **5**, 1–8 (2013).

144. Zhang, J. X. *et al.* Age-related impairment of navigation and strategy in virtual star maze. *BMC Geriatr* **21**, 1–15 (2021).
145. Colombo, D. *et al.* Egocentric and allocentric spatial reference frames in aging: A systematic review. *Neurosci Biobehav Rev* **80**, 605–621 (2017).
146. Kuningas, M., Putters, M., Westendrop R. G., R., Slagboom, E. & van Heemst, D. SIRT1 Gene, Age-Related Diseases, and Mortality: The Leiden 85-Plus Study. *The Journals of Gerontology* **62**, 960–965 (2007).
147. Sciences, N. Roles of the Hippo pathway kinase Ndr2 in neural development and behavior. (2019).
148. Suarez, S. D. & Gallup, G. G. *An Ethological Analysis of Open-Field Behavior in Rats and Mice*. *LEARNING AND MOTIVATION* vol. 12 (1981).
149. Shoji, H., Takao, K., Hattori, S. & Miyakawa, T. Age-related changes in behavior in C57BL/6J mice from young adulthood to middle age. *Mol Brain* **9**, 1–18 (2016).
150. Simon, P., Dupuis, R. & Costentin, J. Thigmotaxis as an index of anxiety in mice. Influence of dopaminergic transmissions. *Behavioural Brain Research* **61**, 59–64 (1994).
151. Xia, Y. *et al.* C/EBPβ is a key transcription factor for APOE and preferentially mediates ApoE4 expression in Alzheimer's disease. *Mol Psychiatry* **26**, 6002–6022 (2021).
152. Hoe, H. S., Harris, D. C. & Rebeck, G. W. Multiple pathways of apolipoprotein E signaling in primary neurons. *J Neurochem* **93**, 145–155 (2005).
153. Konishi, K. *et al.* APOE2 is associated with spatial navigational strategies and increased gray matter in the hippocampus. *Front Hum Neurosci* **10**, (2016).
154. Shinohara, M. *et al.* Apoe2 is associated with longevity independent of alzheimer's disease. *Elife* **9**, 1–16 (2020).
155. Chaudhry, N., Surabhi, S., Mesquita, A. & Riaz, A. Lamp1 mediates lipid transport, but is dispensable for autophagy in Drosophila Resistance to soybean aphids (Aphis glycines) View project RNA metabolism in Drosophila View project. doi:10.1101/2021.03.03.432938.
156. Choi, G. E. *et al.* BNIP3L/NIX-mediated mitophagy protects against glucocorticoid-induced synapse defects. *Nat Commun* **12**, (2021).
157. Kim, S. J. *et al.* Ablation of galectin-3 induces p27 KIP1-dependent premature senescence without oncogenic stress. *Cell Death Differ* **21**, 1769–1779 (2014).
158. Sikora, E. *et al.* Cellular Senescence in Brain Aging. *Frontiers in Aging Neuroscience* vol. 13 Preprint at https://doi.org/10.3389/fnagi.2021.646924 (2021).
159. Xu, M. *et al.* Transplanted Senescent Cells Induce an Osteoarthritis-Like Condition in Mice. *J Gerontol A Biol Sci Med Sci* **72**, 780–785 (2017).
160. Chen, X., Feng, J., Chang, Q., Lu, F. & Yuan, Y. Senescence of donor cells impairs fat graft regeneration by suppressing adipogenesis and increasing expression of senescence-associated secretory phenotype factors. *Stem Cell Res Ther* **12**, (2021).
161. Rin Kim, S. *et al.* Transplanted senescent renal scattered tubular-like cells induce injury in the mouse kidney. *Am J Physiol Renal Physiol* **318**, 1167–1176 (2020).
162. Xu, M. *et al.* Senolytics improve physical function and increase lifespan in old age. *Nat Med* **24**, 1246–1256 (2018).
163. Rattanavirotkul, N., Kirschner, K. & Chandra, T. Induction and transmission of oncogene-induced senescence. *Cellular and Molecular Life Sciences* vol. 78 843–852 Preprint at https://doi.org/10.1007/s00018-020-03638-0 (2021).

164. Poehler, A. M. *et al.* Autophagy modulates SNCA/α-synuclein release, thereby generating a hostile microenvironment. *Autophagy* **10**, 2171–2192 (2014).
165. Bae, E. J. *et al.* TNF-α promotes α-synuclein propagation through stimulation of senescence-associated lysosomal exocytosis. *Exp Mol Med* **54**, 788–800 (2022).
166. Lee, Y. *et al.* Coordinate regulation of the senescent state by selective autophagy. *Dev Cell* **56**, 1512-1525.e7 (2021).
167. Kang, C. *et al.* The DNA damage response induces inflammation and senescence by inhibiting autophagy of GATA4. *Science (1979)* **349**, (2015).
168. Lei, M. *et al.* Trehalose induced by reactive oxygen species relieved the radial growth defects of Pleurotus ostreatus under heat stress. *Appl Microbiol Biotechnol* **103**, 5379–5390 (2019).
169. Luo, Y., Li, W. M. & Wang, W. Trehalose: Protector of antioxidant enzymes or reactive oxygen species scavenger under heat stress? *Environ Exp Bot* **63**, 378–384 (2008).
170. Tang, Q. *et al.* Trehalose ameliorates oxidative stress-mediated mitochondrial dysfunction and ER stress via selective autophagy stimulation and autophagic flux restoration in osteoarthritis development. *Cell Death Dis* **8**, (2017).
171. Sun L *et al.* Trehalose targets Nrf2 signal to alleviate d-galactose induced aging and improve behavioral ability. *Biochem Biophys Res Commun* **1;52**, 113–119 (2019).
172. Doser, R. L. & Hoerndli, F. J. Regulation of neuronal excitability by reactive oxygen species and calcium signaling: Insights into brain aging. *Current Research in Neurobiology* **2**, 100012 (2021).
173. Massaad, C. A. & Klann, E. *COMPREHENSIVE INVITED REVIEW Reactive Oxygen Species in the Regulation of Synaptic Plasticity and Memory*. *Antioxid. Redox Signal* vol. 14.
174. Dhawan, S. *et al.* Reactive Oxygen Species Mediate Activity-Regulated Dendritic Plasticity Through NADPH Oxidase and Aquaporin Regulation. *Front Cell Neurosci* **15**, (2021).
175. Davis, G. W. *et al.* Reactive oxygen species regulate activity-dependent neuronal plasticity in Drosophila. (2018) doi:10.7554/eLife.39393.001.
176. Carter, A. J. R. & Nguyen, A. Q. Antagonistic pleiotropy as a widespread mechanism for the maintenance of polymorphic disease alleles. *BMC Med Genet* **12**, 160 (2011).
177. Cornils, H., Kohler, R. S., Hergovich, A. & Hemmings, B. A. Human NDR Kinases Control G 1 /S Cell Cycle Transition by Directly Regulating p21 Stability . *Mol Cell Biol* **31**, 1382–1395 (2011).
178. Nandakumar, S., Rozich, E. & Buttitta, L. Cell Cycle Re-entry in the Nervous System: From Polyploidy to Neurodegeneration. *Frontiers in Cell and Developmental Biology* vol. 9 Preprint at https://doi.org/10.3389/fcell.2021.698661 (2021).
179. Designed Research; S, L. M. I. I. Rapid initiation of cell cycle reentry processes protects neurons from amyloid-β toxicity. **118**, (2021).
180. Pandey, N. & Vinod, P. K. Model scenarios for cell cycle re-entry in Alzheimer's disease. *iScience* **25**, (2022).

Declaration of Honour

"I hereby declare that I prepared this thesis without the impermissible help of third parties and that none other that the aids indicated have been used; all sources of information are clearly marked, including my own publications.
In particular I have not consciously:

• _fabricated data or rejected undesirable results,
• _misused statistical methods with the aim of drawing other conclusions than those warranted by the available data,
• _plagiarized external data or publications,
• _presented the results of other researchers in a distorted way.

I am aware that violations of copyright may lead to injunction and damage claims by the author and also to prosecution by law enforcement authorities.
I hereby agree that the thesis may be electronically reviewed with the aim of identifying plagiarism.
This work has not been submitted as a doctoral thesis in the same or a similar form in Germany, nor in any other country. It has not yet been published as a whole."

(Place, date)

(Signature)