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Role of AMPK in aging and age-related loss of behavioral plasticity in C. elegans

Caroline Escoubas-Güney

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UNIVERSITÉ CÔTE D'AZUR

ÉCOLE DOCTORALE
SCIENCES DE LA VIE ET DE LA SANTÉ

THÈSE DE DOCTORAT

Role of AMPK in aging and age-related loss of behavioral plasticity in *C. elegans*

Caroline Escoubas-Güney

Laboratoire William Mair / Laboratoire Eric Gilson

Présentée en vue de l'obtention
du grade de docteur en Sciences de la Vie et de la Santé de l'Université Côte d'Azur
Dirigée par : Dr. William Mair
Co-encadrée par : Dr. Eric Gilson
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Eric Gilson, PU-PH, Universite Cote d'Azu
William Mair, Assistant Professor, Harvard University
Colin Ewald, Professor, ETH
Jennifer Tullet, HDR, University of Kent

LA LIGUE CONTRE LE CANCER

Université de Nice Sophia Antipolis – UFR Sciences
Ecole Doctorale Sciences de la Vie et de la Santé

Thèse
Pour obtenir le diplôme de Docteur en Sciences de l'Université de Nice Sophia Antipolis

Role of AMPK in aging and age-related loss of behavioral plasticity in *C. elegans*

(Le rôle de l'AMPK dans le vieillissement et la perte de plasticité neuronale liée au vieillissement chez *C. elegans*)

Caroline Escoubas-Güney
2018

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“Your profession is not what brings home your weekly paycheck, your profession is what you're put here on earth to do, with such passion and such intensity that it becomes spiritual in calling”

Vincent Van Gogh

Acknowledgements

My PhD was a journey. A journey filled with ups and downs, with dreams, hopes, and excitement for science as well as disappointments, doubts, and solitude, but never regrets. I truly believe that interrupting my medical studies to pursue a PhD was the best decision I ever made.

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I would like to thank the Ligue Nationale Contre le Cancer and Groupe Pasteur Mutualité for funding my work.

Declaration

I declare that the work presented in this thesis is my own except where duly noted.

Caroline Escoubas

Abstract - English

The dramatic increase in life expectancy during the 20th century was accompanied by a resultant epidemic of age-related pathologies including neurodegenerative diseases. Unfortunately, current therapeutics primarily focusing on protein misfolding aspects of diseases such as Alzheimer's Disease (AD) have been unsuccessful in the clinical trials. Recent epidemiological studies have suggested a strong association between metabolic dysfunction and neurodegeneration. Therefore, an alternative approach is to target metabolic pathways disrupted in AD models for therapeutics. AMP activated protein kinase (AMPK) is activated in a low energy state via sensing the AMP:ATP ratio. Once active, AMPK promotes longevity in model organism and protects against a wide range of age related diseases including neurodegenerative diseases. In addition, AMPK regulates mitochondrial homeostasis and mitochondrial networks in mammals. However, whether mitochondrial regulation causally links AMPK to protection against neurodegenerative disease is unknown. Here we use a learning and memory protocol in *C. elegans* as readout of neuronal function. We show that nematodes expressing the toxic amyloid peptide $A\beta^{1-42}$ in the neurons display impaired learning ability, which can be rescued by constitutive activation of AMPK (CA-AMPK). We further show that CA-AMPK enhances learning ability in wild type nematodes by promoting mitochondrial fusion. Indeed, fusion deficient worms show impaired learning, which can be rescued by restoring mitochondrial fusion specifically in the neurons. Additional results suggest that AMPK might promote its beneficial effects on neuronal function via inhibition of CREB-regulated transcriptional co-activator 1 (CRTC-1). Our results show that targeting neuronal metabolism may be a viable therapeutic option to restore neuronal function in the context of neurodegenerative diseases.

Key words: aging, *C. elegans*, Alzheimer's disease, associative learning, AMPK, mitochondrial dynamics

Résumé - Français

La progression de l'espérance de vie observée au cours du XXième siècle a été accompagnée par une augmentation massive de l'incidence des maladies liées à l'âge et en particulier des maladies neurodégénératives. Malheureusement, les thérapeutiques actuelles ciblant principalement les anomalies d'agrégation protéique caractérisant ces maladies, tel que la maladie d'Alzheimer, ont échoué au niveau des essais cliniques. De récentes études épidémiologiques ont suggéré un lien entre la dysfonction métabolique et les maladies neurodégénératives. Par conséquent, une approche alternative pour développer des nouveaux médicaments serait se cibler les voies de signalisation métaboliques perturbées dans les modèles de maladie d'Alzheimer. L'AMPK (AMP activated protein kinase) est une enzyme activée par les bas niveaux d'énergie cellulaire via la détection du taux AMP:ATP. Une fois activée, l'AMPK allonge la durée de vie d'organismes modèles et protège contre le développement de pathologies liées à l'âge telle que les maladies neurodégénératives. De plus, l'AMPK régule l'homéostasie mitochondriale et les réseaux mitochondriaux chez les mammifères. Cependant, il reste à savoir si l'AMPK protège contre le développement de pathologies neurodégénératives via la régulation de la structure mitochondriale. Lors de ces travaux, nous avons utilisé un protocole d'apprentissage et de mémoire chez *C. elegans* pour mesurer la fonction neuronale. Nous avons montré que les nématodes exprimant le peptide amyloïde Aβ^{1-42} dans les neurones avait une capacité d'apprentissage détériorée. Ce déficit a pu être restauré par l'activation constitutionnelle de l'AMPK. Nous montrons également que l'activation de l'AMPK améliore les capacités d'apprentissage des nématodes sauvages en induisant la fusion des mitochondries. En effet, les vers mutés pour le gène responsable de la fusion mitochondriale ont une capacité d'apprentissage diminuée, laquelle peut être restaurée par le rétablissement de la fusion mitochondriale, spécifiquement dans les neurones. Des résultats supplémentaires suggèrent que l'AMPK induirait ses effets bénéfiques sur la fonction neuronale en inhibant le facteur de transcription CRTC-1 (CREB-regulated transcriptional co-activator 1). Nos résultats tendent à montrer que cibler le métabolisme cellulaire neuronal représenterait une option thérapeutique viable afin de maintenir les fonctions neuronales dans le cadre de pathologies neurodégénératives.

Mots clés: vieillissement, *C. elegans*, maladie d'Alzheimer, mémoire associative, AMPK, dynamique mitochondriale

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List of abbreviations

ALS: amyotrophic lateral sclerosis

AMPK: AMP activated protein kinase

AD: Alzheimer's disease

C. elegans: Caenorhabditis elegans

CREB: cAMP response element binding protein

CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats

CRTC: CREB regulated transcription coactivator

DR: dietary restriction

DRP-1: dynamin related protein 1, *C. elegans* homolog of DRP1

FZO-1: fuzzy onion 1, *C. elegans* homolog of MFN1/2

GFP: green florescent protein

HD: Huntington's disease

ND: neurodegenerative disorders

PD: Parkinson's disease

S6K: ribosomal protein S6 kinase

SFA-1: splicing factor 1

TOR: target of rapamycin

1. Introduction

1.1. The challenges of an aging population

1.1.1. Aging as a risk factor for chronic diseases

Dramatic increases in life expectancy was one of the most distinctive demographic events of the 20th century, with 27 years added to average longevity due to advances in medicine and public health measures. In 1955, worldwide average life expectancy was 46 years old, but by 2015 this number had risen by nearly 20 years to 65 (United Nations Report, 2015). This increase in world life expectancy is projected to continue, as developing countries improve their health care systems. By 2100, at least half of the human population worldwide can expect to live to 83 years of age (United Nations Reports, 2015). Increased life expectancy will inevitably mean a change in demographic, with a significant percentage of the population being over the age of 65 an estimated 1.5 billion people by 2050 (United Nations Report, 2015). However, a downside to the aging population in the resultant epidemic of age-related pathologies, leaving a burden on the health care

system which is rapidly becoming untenable. Indeed, overall healthcare expenses are expected to exceed 500 billion dollars in the US by 2100 (**Fig1.1**).

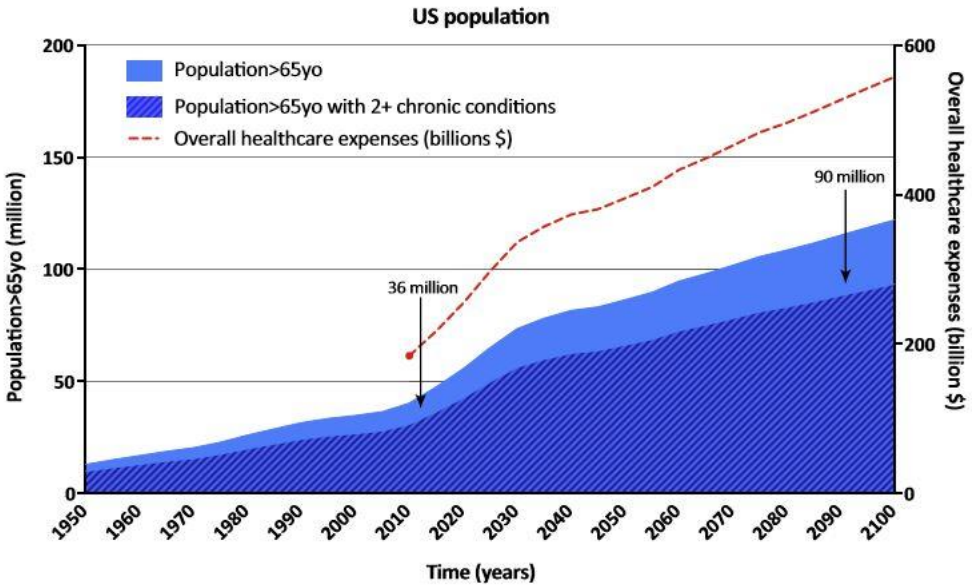

Figure 1.1: US population >65yo represents both a public health and economic burden[1]

Age is the biggest risk factor for the vast majority of chronic diseases including some of the biggest killers, such as metabolic diseases, cardiovascular disease and neurodegenerative disorders. Worse still, many of these conditions often occur simultaneously in the same individual – in the USA over half of people older than 65 years have two or more chronic conditions (**Fig1.2**). Current therapeutic strategies rely on understanding the underlying mechanisms of individual diseases such as cancer, heart failure or dementia in order to cure them. Unfortunately, curing one

condition will only provide a limited increase in the "disease-free" lifespan of an elderly individual as this person will most likely continue to suffer from multiple other age-related conditions.

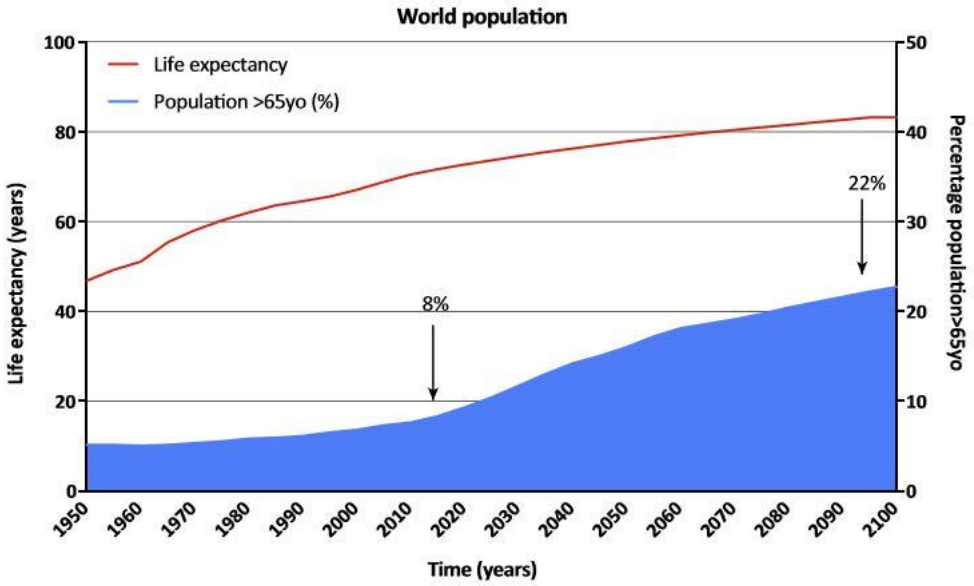

Figure 1.2: Projected life expectancy and percentage of aging population in the world[1]

An emerging alternative approach is that of 'geroscience'[2]. Instead of focusing on proximal mechanisms of individual age-related diseases in isolation, geroscience focuses on aging as a common risk factor, and on the cellular components that might link patient age to overall disease risk. Although aging research is a rather young field, several genetic, environmental, and pharmacological interventions that slow aging and decrease age-related pathology of model organisms have been characterized. Among those, Dietary Restriction or "reduction of food intake without malnutrition" is a very potent intervention for lifespan extension. Decreasing food intake

during dietary restriction (DR) increases longevity in species ranging from yeast to primates[3] and has protective effects against multiple age-onset conditions. Conserved mechanisms that integrate upstream energy-sensing pathways with targeted downstream transcriptional targets might therefore be ideal targets to specifically recapitulate the physiological benefits of DR for therapeutic purposes.

1.1.2. Alzheimer's Disease and the need to find new targets

Neurodegeneration, and in particular dementia, is one of the biggest contributors to age related diseases and its increasing prevalence represents a major burden as these patients display increased susceptibilities to other comorbidities and usually become dependent on care-givers many years before death[4]. The most prevalent neurodegenerative disorder is Alzheimer's Disease (AD), a disease which currently affects 5.5 million Americans. Given the growing proportion of elderly people in the general population (**Fig1.2**), an estimated 16 million Americans will suffer from AD by 2050. AD is a leading cause of disability and poor health, representing the 6th most prevalent cause of death in the USA and effectively costing 259 billion dollars in health care in 2017 alone (www.alz.org). AD is a progressive neurodegenerative disorder characterized by a

decay in cognitive abilities and in particular learning and memory. Patients also exhibit spatial and temporal disorientation, changes in mood and behavior and at the later stages of the disease, difficulties speaking or walking, all of which leading to a loss of autonomy (www.alz.org). Brains of individuals suffering from AD display extracellular accumulation of amyloid fibrils and plaques made of the toxic amyloid peptide Aβ, hyperphosphorylation of intracellular tau and neuronal cell death[5]. The amyloid cascade hypothesis stipulates that AD pathogenesis is caused by the abnormal processing of APP (Amyloid Precursor Protein) thus leading to the formation and accumulation of the toxic amyloid peptide Aβ[6-8]. APP is synthetized and transferred to the plasma membrane through the ER-Golgi secretory pathway, where it can then be processed through the amyloidogenic or non-amyloidogenic pathways (**Fig1.3**). Through the non-amyloidogenic pathway, APP is cleaved at the plasma membrane by α-secretases resulting in an extracellular fragment, the soluble APPα, and an intracellular fragment CTFα (C-Terminal Fragment). The CTFα can be hydrolyzed inside the cytoplasm by γ-secretases to release the transcription factor peptide AICD (APP intracellular domain), the physiological role of which is still not fully understood. Binding of cytoplasmic membrane APP to low density lipoprotein receptors such as

SORL1 leads to its internalization in recycling endosomes[9]. This physiological processing of APP does not result in formation of toxic amyloid peptides and is thus called the nonamyloidogenic pathway. When processed through the amyloidogenic pathway, cytoplasmic membrane bound APP is internalized into late endosome where it is cleaved by the β-secretase (BACE), releasing the N-terminal sAPPβ region into the endosome and leaving the C-terminal fragment CTFβ in the membrane. CTFβ is then hydrolyzed at the membrane by the γ-secretase complex containing the presenilin enzymes, yielding the toxic peptides $A\beta^{1-40}$ or $A\beta^{1-42}$ depending on the cleavage site. These amyloid peptides are released in the extracellular space where they aggregate to form oligomers or fibrils and plaques, disrupting synaptic functions by binding to cytoplasmic membrane bound receptors and ion channels and inducing a constant state of inflammation in the brain[5,10]. The brain can physiologically degrade amyloid peptides either through autophagy of the Aβ-containing endosomes[11] or through microglia-mediated phagocytosis of extracellular Aβ and IDE (insulin-degrading enzyme)-mediated degradation. Apolipoprotein E plays a prominent role in this amyloidogenic pathway as it can influence both the IDE-mediated degradation and the

endocytosis process, explaining why polymorphisms of the APOE gene, such as ApoE4 isoform, can increase susceptibility to develop AD[13,14].

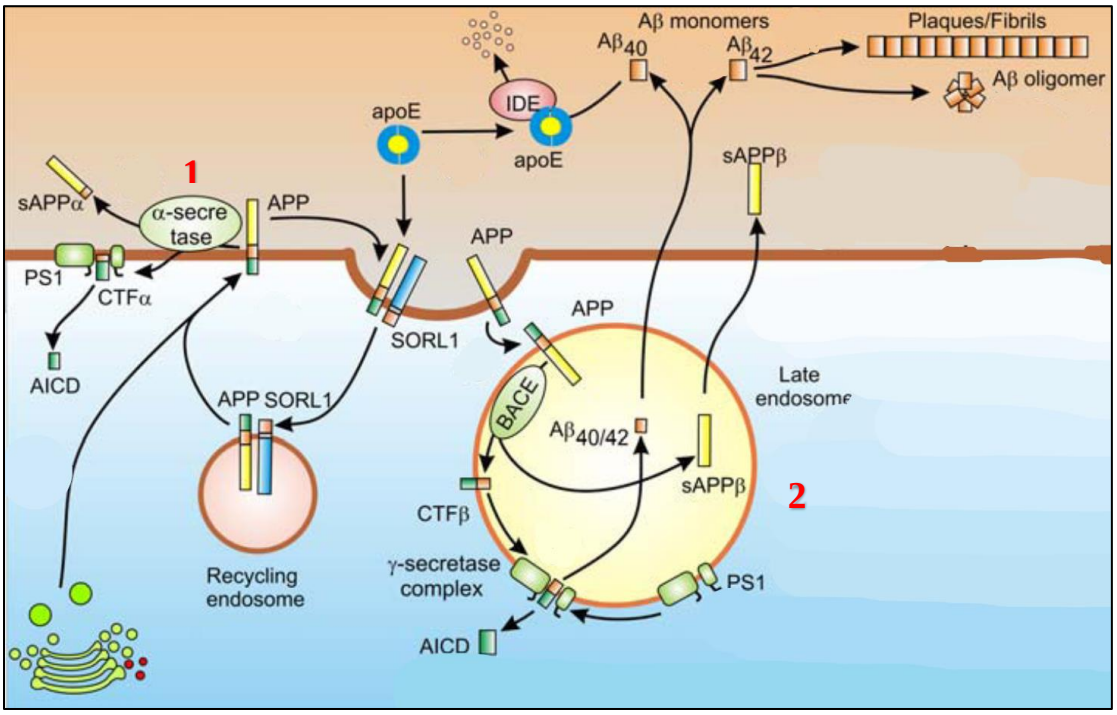

Figure 1.3: Amyloidogenic (2) vs. non-amyloidogenic (1) pathways. Adapted from Berridge *et al.*[12]

Despite strong evidence that Aβ accumulation in the brain, as stated by the amyloid cascade hypothesis, is responsible, or at least tightly linked, to the onset of AD, an increasing number of alternative hypotheses have emerged to explain the pathogenesis of AD, as it is still unclear how Aβ oligomers alter neuronal function and cause neurodegeneration. One strong argument in favor of the amyloid cascade hypothesis is that the autosomal dominant familial form of AD,

characterized by early onset cognitive decline (<60 years old), is caused by mutations in key components of the amyloidogenic pathway such as APP, ApoE4, presenilin 1-2 and SORL1. These mutations directly link Aβ metabolism to the rapid onset of AD symptoms[15-18]. Interestingly, Down's Syndrome (or Trisomy 21) patients display accumulation of AD-like amyloid plaques, a phenotype which could be linked to the extra copy of APP gene they carry on chromosome 21[19]. In the last two decades, AD-related research has represented almost 18% of neuroscience research efforts[20] but unfortunately, since 2003, neither a new medication or an effective prevention method has been approved by the FDA for AD[21] (www.alz.org). Research has focused on the amyloid cascade hypothesis as the main driver of AD pathogenesis and although targeting components of the amyloidogenic pathway has yielded promising results in animal models, it has failed in human clinical trials[22].

Several observations challenge the Aβ centered view of AD: the brains of some cognitively healthy human subjects have been described to contain amyloid plaques similar to those normally encountered in AD patients, and Aβ actually accumulates long before clinical symptoms manifest[23,24]. Furthermore, neuronal accumulation of neurofibrillary tangles of hyper-

phosphorylated tau, although present in other forms of dementia[25], actually better correlate with degradation of cognitive functions[24]. It has therefore become apparent that alternative hypotheses have to be explored to determine novel therapeutic targets to test in clinical trials. Since 2009, several GWAS studies have substantiated this idea by identifying over 20 different SNPs across the genome mediating AD risk, involved in various cellular functions such as inflammation, lipid metabolism or endocytosis[26]. Among those alternative hypotheses regarding AD pathogenesis are i) the calcium hypothesis, linking amyloid metabolism to neuronal dysfunction[12,27-30], ii) the bioenergetics hypothesis, stating that the switch from a glucose-dependent to a ketone-based metabolism or fatty acid oxidation is accompanied in the brain by oxidative stress, decline in mitochondrial function, calcium overload, and general cellular malfunction[31], iii) the inflammation hypothesis, proposing chronic brain inflammation as the initiator of AD[32], and iv) the microglial hypothesis, suggesting that Aβ induced alteration in glial cells contribute to impaired glutamate transport, calcium dysregulation and release of pro-inflammatory cytokines[33].

Since current therapeutics primarily focusing on the protein misfolding aspect of AD have been unsuccessful in clinical trials so far, in my thesis, I decided to explore an alternative pathway

which could participate in the pathogenesis of the disease. Epidemiological studies have shown that obese or diabetic patients are at higher risk of developing AD, suggesting a strong association between metabolic dysfunction and neurodegeneration[34]. Furthermore, animal models have provided insight into neuronal metabolic dysregulation observed in AD and have demonstrated the critical causal role of mitochondrial dysfunction leading to neuronal dysfunction and cellular death[35]. An alternative AD therapeutic approach might therefore be to target metabolic pathways disrupted in AD, rather than targeting the protein folding pathways or the ER stress response induced by accumulation of toxic aggregates. To explore this hypothesis, I decided to focus on dietary restriction, an intervention which not only extends lifespan but reduces the risk of developing age-related diseases including neurodegenerative disorders by modulating cellular metabolism.

1.1.3. Benefits of dietary restriction in the aging brain

McCay and colleagues were the first to demonstrate that caloric restriction (CR) could lead to lifespan extension in rats compared to *ad libitum* fed rats[36]. This important finding was then extensively reproduced with different DR protocols and showed lifespan extension in almost all

model organism tested as well as promotion of healthy aging[3,37]. Throughout this dissertation, we will use the term Dietary Restriction (DR) for any intervention with reduces food intake, including caloric restriction. DR promotes healthy aging by delaying the onset or improving the outcome of major age-related chronic diseases such as cancer, cardiovascular disease metabolic disorder or neurodegenerative diseases[38].

Besides its beneficial effects on lifespan and the general physiology of the organism, modulation of diet can impact brain structure, plasticity and function and affect markers of brain physiology such as synaptic function, trophic factors or hippocampal neurogenesis[39,40]. The hippocampus, a brain region known to be highly sensitive to environmental changes and considered to be central in regulating learning and memory processes can be modulated by dietary restriction suggesting potential benefits on cognitive functions[41-43]. DR seems to benefit neuronal plasticity by improving synapse adaptation to oxidative or metabolic stress[44], potentially through differential expression of genes implicated in synaptic plasticity[45]. In addition, DR stabilizes the levels of glutamate receptors and synaptic proteins required for excitatory transmission, a key process in hippocampal mediated memory formation[46,47]. Finally, long term DR in mice improves

working memory, suggesting that the cellular changes induced by DR can translate into cognitive benefits[48,49]. Similar beneficial effects have been reported upon intermittent fasting (alternate periods of *ad libitum* intake with complete or partial restriction of calories) suggesting that other interventions that modulate diet can impact cognitive outputs[50].

Given that the aging brain is characterized by metabolic changes, including increased vulnerability to toxic or metabolic insults eventually leading to impaired synaptic function such as long-term potentiation[51,52], a number of researchers have explored the possibility that DR mediated cellular changes in the normal brain could delay or reverse age related neuronal decline. The benefits of DR on the aging brain include the prevention of age related decline in learning and the preservation of spatial and working memory in mice upon late onset DR[53,54]. Similar improvements can be observed with either midlife onset DR[55] or late stage short exposures to DR[56,57]. The mechanisms of these effects could be explained by the prevention of age-related loss of synaptic proteins[58] or by DR-induced adult neurogenesis[59]. These results are very interesting

for potential translation to humans although a life-long DR regimen is not an easily sustainable therapeutic or preventative measure in humans.

These data linking dietary restriction to healthy brain aging have prompted health care authorities to test the translational potential to humans. Two major population studies have been initiated: the National Institute of Aging CALERIE trial (Comprehensive Assessment of Long-Term Effects of Reducing Intake of Energy) and the ENCORE study (Exercise and Nutrition Interventions for Cardiovascular Health)[60,61]. Although the 6 months DR regimen experimented in the CALERIE trial did not lead to improvement of cognitive functions, the ENCORE study was able to demonstrate enhanced executive-function learning when DR was combined with exercise[62]. Moreover, Witte *et al.* reported that a daily 30% DR regimen in a population of elderly subjects (>60yo) improved by ~20% the verbal memory scores compared to an *ad libitum* group[63]. This is the first study to show an added benefit of DR on cognitive function in an elderly human population and therefore opens new questions for potential beneficial effects of DR on age-related brain disease prevention or modification of pathogenesis.

Dietary interventions have been reported to enhance cognitive or neuronal functions not only in the normal aging brain but also in the context of mood changes or anxiety and neurodegenerative diseases such as Alzheimer's disease[50]. Aβ mediated synapse dysfunction is a potential key contributor to the disease and it has therefore been hypothesized that DR could alleviate AD pathogenesis by preserving synaptic function[64,65]. A 30% DR diet was able to alleviate memory deficits and pathophysiology of the disease in a mouse AD model, likely through the upregulation of genes associated with neurogenesis, synaptic plasticity and downregulation of inflammatory markers[66]. These results have the potential to be translated to humans as epidemiological studies demonstrate an association between high caloric diets and risk of developing AD or suffering from mild cognitive impairment[67-69]. Likewise, intermittent fasting, another dietary intervention shown to extend lifespan, started prior to the onset of the disease phenotype, can rescue the age-related cognitive decline in 17 months old 3xTg-AD mice[70] as measured by the Morris water maze assay[71].

Unfortunately, DR induces a number of detrimental side effects on growth, reproductive capacity, immunity or wound repair that may limit its translational potential to humans[72]. In order

to harness the beneficial effects of DR without suffering its negative side effects, we need to elucidate pathways underlying the effects of DR on aging and more specifically in the brain. Uncovering new cellular mechanisms will provide novel targets which can potentially be targeted pharmacologically for treatment of human diseases such as AD. DR can be performed in various ways in laboratory settings and even though different protocols of reduction of food intake can extend lifespan, they might not all rely on the same genetic pathways[3]. However, most DR mediators are metabolic regulators involved in nutrient signaling and nutrient sensing pathways such as insulin, sirtuins or TOR/AMPK (Target of Rapamycin, AMP activated protein kinase)[3].

Given the well-documented role of AMPK in the context of aging and age-related neurodegenerative diseases, we focused our work on the role of the central energy sensor and metabolic regulator, AMPK, as a downstream mediator of DR. Indeed, the aging process has been associated with an alteration of AMPK signaling which normally plays a key role in maintaining cellular homeostasis in response to various stresses. Evidence shows an age-dependent reduction in AMPK Thr-172 phosphorylation levels in myocardium, skeletal and endothelial tissue[73], as well

as changes in expression levels of the different subunits required to form the AMPK complex (α,β,γ)[74], whereas other studies point towards an alteration of AMPK capacity to respond to stress in old animals[73]. The aging process is associated with a decline in AMPK activation in old versus young rat muscles in response to activation insults[75] or electrical stimuli[74]. Similarly, AMPK's response to stroke in the brain or hypoxia in the liver is lost in old mice. These observations in mice correlate with data in the model organism the nematode *Caenorhabditis elegans*[76], in which the AMP:ATP ratio can be used as a lifespan predictor[77]. Increasing the expression of AMPK catalytic subunit (*aak-2* gene) is sufficient to increase lifespan[77] and similar lifespan extensions in *C. elegans* have been reported via transgenic expression of a constitutive active form of AMPK generated by a mutation in the AMP-binding site mutant of the regulatory subunit γ[78] or expression of the truncated catalytic subunit AAK-2 (aa 1-321)[79]. This effect is also conserved in *Drosophila,* where overexpression of the catalytic subunit of AMPK in muscle or fat body extends lifespan[80].

1.2. Targeting AMPK in the context of neurodegeneration

1.2.1. AMPK structure

The AMP-activated protein kinase (AMPK) is a highly conserved key sensor of cellular energy status, present in essentially all eukaryotic cells[81-84]. Cellular energy deficiency, as characterized by ATP depletion, activates AMPK, which in turn inhibits anabolic reactions and stimulates catabolic reactions in order to increase energy production and decrease cellular ATP usage. AMPK is a heterotrimeric complex which consists of an α catalytic subunit and β and γ regulatory subunits[85]. In mammals, these subunits have several isoforms, which are differently expressed in different tissues. Phosphorylation of the α catalytic subunit at the Threonine-172 residue (Thr-172) is required for the activation of AMPK[86] and induces its kinase activity greater than 100-fold. Three upstream kinases target this phosphorylation site to activate AMPK: Liver Kinase B1 (LKB1) which is predominant, especially in the context of energy stress[87-90], Ca^{2+}/Calmodulin-dependent kinase kinase β (CAMKKβ)[91,92] and transforming growth factor-β-activated kinase 1 (Tak1)[93,94]. On the other hand, the phosphorylated Thr-172 residue can be de-phosphorylated by three AMPK phosphatases: Protein Phosphatase 1 (PP1), Protein Phosphatase

2A (PP2A) and Protein Phosphatase 2C (PP2C)[95-97]. The activity of AMPK is not only dependent on upstream stimulatory and inhibitory signals but is also dependent on cellular energy status, as nucleotide binding acutely tunes AMPK activation. The γ regulatory subunit contains four potential nucleotide-binding sites, which can either bind AMP, ADP or ATP. Under low energy conditions when the AMP/ADP:ATP ratio increases, AMP and ADP compete with ATP to bind AMPK[85]. Three consequences arise from this nucleotide binding: allosteric activation of AMPK 2-10 fold, promotion of Thr-172 phosphorylation by upstream kinases[98] and inhibition of Thr-172 de-phosphorylation by protein phosphatases most likely through conformational changes[85,99]. The binding of ATP antagonizes these effects, therefore making AMPK a very sensitive sensor of AMP:ATP and ADP:ATP levels.

1.2.2. AMPK downstream targets

When activated, AMPK modulates downstream signaling pathways in order to replenish cellular ATP supplies on one side and reduce ATP-consuming biosynthetic processes on the other side. Studies of AMPK downstream targets led to the identification of a conserved sequence motif surrounding the Serine/Threonine residue phosphorylation by AMPK[100]. Basic residues at -4 or -

3 positions relative to the phospho-acceptor site and hydrophobic residues at -5 and +4 positions seem to be important for the motif recognition[100-103]. In order to generate more ATP, AMPK enhances glucose uptake in muscles by inducing the translocation of the glucose transporter GLUT4 to the plasma membrane[82]. The intracellular storage vesicles containing GLUT4 require the RAB family of proteins to be GTP-bound[104]. In the contracting muscle, AMPK phosphorylates the RAB-GAP protein TBC1D1, which normally maintains the RAB proteins in the inactive GDP-bound state. Phosphorylation by AMPK induces its association with 14-3-3 protein and subsequent dissociation from the vesicles, triggering the conversion of the RAB protein to its active GTP-bound form and the fusion of the GLUT4 carrying vesicles to the plasma membrane[104-107]. In addition to GLUT4, AMPK also promotes glucose entry via activation of another plasma membrane-located glucose transporter, GLUT1, expressed in most cells other than muscle, liver and adipose tissue[108]. Finally, AMPK activates glycolysis via phosphorylation of PFKFB (PFKFB2 isoform, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase) in cardiac myocytes or monocytes and macrophages. Active phosphorylated PFKFB in turn, catalyzes the generation of fructose-2,6-bisphosphate, the allosteric activator of the glycolytic enzyme PFK1 (6-

phosphofructo-1-kinase)[109,110]. In addition to upregulating glucose catabolism, AMPK inhibits its anabolism by inhibiting the glycogen synthesis enzyme, glycogen synthase[111] as well as other gluconeogenic enzymes such as phospho-enolpyruvate carboxykinase and glucose-6-phosphatase[112,113]. AMPK downregulates the expression of these gluconeogenic enzymes by phosphorylating and excluding from the nucleus the transcription factor CRTC2 (CREB-regulated transcription co-activator 2)[1] and the class IIa HDAC (histone deacetylase), which normally deacetylates and activates the FOXO transcription factors[113]. Regarding lipid metabolism, AMPK, on one hand, upregulates fatty acid uptake and β-oxidation and on the other hand downregulates triglycerides and fatty acids synthesis pathways and lipogenesis. AMPK promotes fatty acid uptake first in the cell, as shown in cardiomyocytes via translocation of CD36-containing vesicles[114] and second, into the mitochondria, by phosphorylation and subsequent inactivation of ACCβ (acetyl-CoA carboxylase), leading to a reduction in the inhibitor of mitochondrial fatty acid entry, malonyl-CoA[115]. AMPK phosphorylates and inhibits the lipogenic transcription factor SREBP1c (sterol regulatory element binding protein)[116]. In addition to this master regulator of lipogenesis, AMPK targets other enzymes such as acetyl-CoA carboxylase 1 (ACC1), glycerol phosphate acyl-

transferase (GPAT) or 3-hydroxy-3-methylglutaryl CoA reductase (HMGR) in order to inhibit fatty acid synthesis, triglyceride and phospholipid synthesis or cholesterol synthesis respectively[117-119]. AMPK modulates protein metabolism by reducing protein synthesis on one side and increasing protein degradation through autophagy on the other side. AMPK represses protein synthesis both directly, by phosphorylation of eEF2 (eukaryotic elongation factor 2) upstream inhibitory kinase[120] and indirectly, by inhibiting the mTOR pathway through phosphorylation of TSC2 (tuberous sclerosis 2) or RAPTOR (regulatory-associated protein of mTOR)[121,122]. Subsequent inhibition of mTOR downstream targets S6K and 4E-BP impacts translation initiation, therefore affecting protein synthesis[123]. AMPK also acts at the transcriptional level by inhibiting the transcription factor TIF-IA (transcription initiation factor IA), responsible for the expression of the rRNA producing enzyme, RNA Polymerase I[124]. Recycling of organelles and cytosolic content through autophagy is a general catabolic response to starvation, also activated by AMPK, both in mammals and in yeast[125]. AMPK is able to form a stable complex with ULK1/2 proteins (UNC-51-like kinase 1)[126] and phosphorylate them[127,128]. This triggers autophagy through the activation of VPS34 and subsequent cellular trafficking events[129]. As a second layer of regulation,

AMPK has been shown to phosphorylate Beclin-1 to promote the formation of "pro-autophagy" VPS34 complex at the expense of "non-autophagy" VPS34 complexes involved in intracellular vesicle trafficking[130].

Loss of mitochondrial homeostasis has been correlated with the aging process and it is therefore not surprising to find interactions between AMPK and mitochondrial biology. AMPK drives the switch from carbohydrates to lipids as the main energy source in response to fasting and exercise in skeletal muscle cells[131]. AMPK transcriptionally induces mitochondrial biogenesis by modulating the activity of the "master regulator" of mitochondrial biogenesis, PGC-1α (Peroxisome proliferator-activated receptor Gamma Coactivator-1 α), a transcription factor which enhances the expression of nuclear-encoded mitochondrial genes[132]. AMPK directly phosphorylates PGC-1α, which leads to its own transcription through a positive feedback loop[133]. A second mechanism has been described, where AMPK promotes SIRT1 (sirtuin1) activity by increasing the cellular concentrations of NAD^+, leading to increased deacetylation and activation of PGC-1α[131,134]. In addition to mitochondrial biogenesis, AMPK controls the turnover of damaged

mitochondria, a process known as mitophagy. Indeed, inhibition of ULK1 phosphorylation by AMPK leads to the accumulation of mitochondria with abnormal morphology and reduced membrane potential, suggesting that AMPK has a role in maintaining efficient, ATP-generating, mitochondria by removal and recycling of the damaged mitochondria[127]. Finally, it was more recently demonstrated in our laboratory that AMPK regulates mitochondrial morphology (fusion and fission process) to maintain mitochondrial network homeostasis during aging and preserve functional coordination with peroxisome to promote fatty acid oxidation[135]. As a consequence of this regulation, loss of muscle AMPK activity in a murine model (AMPKβ1/AMPKβ2 double knockout) leads to reduced mitochondrial content and diminished muscle performance[136], whereas activation of AMPK via AICAR (5-AminoImidazole-4-CArboxamide Ribonucleotide) increases mitochondrial genes expression in muscles and confers beneficial effects in term of exercise endurance to "sedentary mice"[137,138].

1.2.3. AMPK neuronal functions

An increasing number of publications suggest a very prominent role for AMPK both for the normal function of neuronal tissue and in the context of cognitive impairment and

neurodegenerative diseases[76]. A cross sectional population study in Korean subjects aged >60 years old shows a significant association between the AMPK γ2 gene polymorphism (PRKAG2 -26C/T) and cognitive impairment in old age, independent of the presence of diabetes. Consistent with the hypothesis that metabolic dysregulation is tightly link to cognitive dysfunction, this SNP has previously been associated with diabetes[139]. While the role of AMPK as energy sensor and metabolic regulator in tissues like liver, muscle and adipose tissue has been described, it is also present in the mammalian brain [140-142] and its functions in the nervous system and more specifically in memory formation have only recently gained attention[143-145]. It seems intuitive to hypothesize an important role for AMPK in neurons due to the fact that the brain combines a high metabolic rate with a poor nutrient storage capacity, making it vulnerable to large energy variations. AMPK is a modulator of Long Term Potentiation (LTP)[146], and has been shown to increase NMDA-induced K-ATP currents by a Ca^{2+}-dependent process, which as a consequence diminishes the excitatory effect of glutamate-mediated transmission in rat neurons[147]. Further characterizing the synaptic role of AMPK, a group showed a decrease in the levels of LKB1 – an AMPK upstream activator – and active AMPK in old mice[148]. Pointing to an important function of AMPK in synapse

formation, deletion of either kinase led to aberrant axonal retraction, extension of postsynaptic dendrites and formation of ectopic synapses in retinal neurons whereas genetic and pharmacological (metformin, caloric restriction) activation of AMPK protects against age-related synaptic alterations in those neurons[148]. Consistent with its role as a key metabolic modulator, AMPK has protective effects against metabolic and excitotoxic insults. The AMPK agonist AICAR protects rat hippocampal neurons from various stresses such as glucose deprivation, chemical hypoxia, glutamate exposure and Aβ toxicity[141]. GABA (γ-AminoButyric Acid), the major inhibitory neurotransmitter in the brain, might mediate AMPK's protective effect. AMPK activates $GABA_B$ receptors which then modulate post-synaptic K^+ signaling as well as pre-synaptic Ca^{2+} channels, to suppress neuronal excitation, thus exerting a neuroprotective effect during metabolic insults[149,150].

Despite these publications, the beneficial or detrimental role of AMPK in neuronal function still needs to be further addressed, as there is evidence of a deleterious role of AMPK activation in the context of stroke damage in a murine model of cerebral artery occlusion[151]. Highlighting this discrepancy, pharmacological activation of AMPK by AICAR can increase spatial memory in

young and old mice and correlates with the induction of neuronal development and plasticity genes[152], or impair the formation of long term fear memory[142]. Indeed, a decrease in AMPK activity following contextual fear conditioning is required for fear memory formation[142]. These discrepancies might be explained by the different mechanisms involved in the formation of spatial versus fear memory. AMPK proved to have an even more prominent role in the context of neurodegenerative diseases and more specifically Alzheimer's Disease. AMPK is required downstream of two interventions known to ameliorate the pathology of Alzheimer's Disease: leptin[153] and resveratrol[154]. Inhibition of Aβ production and tau phosphorylation by leptin and extracellular Aβ accumulation by resveratrol in neuronal cultures both required AMPK and could be replicated using pharmacological activators of AMPK[153,154]. Likewise, AMPK activation inhibits palmitate-induced apoptosis and tau hyperphosphorylation in SH-SY5Y cells[155]. Contradicting with the potential beneficial role of AMPK in Alzheimer's Disease, AMPK is a tau kinase, which could potentially contribute to disease pathogenesis by altering microtubule binding of tau[156]. AMPK abnormally accumulates in tangle and pre-tangle bearing neurons in major tauopathies and it was shown *in vitro* that $A\beta^{1-42}$ induced activation of AMPK via CaMKKβ

(Ca^{2+}/calmodulin-dependent protein kinase kinase β) leads to increased phosphorylation of tau at Ser^{262}/Ser^{356}/Ser^{396}[156,157]. These observations were confirmed *in vivo* in an AMPKα2 deficient mouse model, which displayed reduced levels of endogenous tau phosphorylation[158]. Supporting a contribution to the pathology of Alzheimer's Disease, AMPK activation, through the transcriptional upregulation of BACE1, significantly increases the generation of intracellular and extracellular Aβ peptides[159]. Despite convincing evidence that AMPK is a tau kinase, these findings do not address the functional impact of such phosphorylation *in vivo* on cognitive functions. It is therefore difficult to assess whether this increased tau phosphorylation by AMPK contributes to the memory loss or neurodegeneration observed in Alzheimer's Disease. *Ma et al*. sought to study the effect of AMPK inhibition on long term potentiation (LTP) and long-term depression (LTD) in APP/PS1 transgenic mice, a process considered as the cellular basis for learning and memory formation. They showed a negative contribution of AMPK as treatment of this murine Alzheimer's model with the AMPK inhibitor compound C or genetic deletion of the AMPK catalytic subunit prevented Aβ induced LTP loss[160]. This once again contrasts with the

behavioral data clearly showing beneficial effects of activation of AMPK on memory formation[161,162].

These discrepancies between the behavioral and the cellular effects of AMPK in neurons point to the gap in the understanding of the mechanisms of action of AMPK and the need to further characterize the downstream targets of AMPK mediating its behavioral benefits. Further highlighting the complex role of AMPK in cognitive functions, polyQ-expanded mutant huntingtin (HTT) protein causing Huntington's Disease induces abnormal activation of AMPK in both human and mice striatum neurons. The aberrant AMPK activation contributes to the progression of the disease by increasing oxidative stress, brain atrophy, neuronal loss and mutant HTT aggregation[163,164]. Similarly, in Parkinson's Disease cellular models or *Drosophila* models, AMPK activation was both characterized as detrimental[165] or beneficial[166,167] in disease pathogenesis as measured by neuronal cell death. Modulation of AMPK in amyotrophic lateral sclerosis (ALS), another neurodegenerative disease caused by selective loss of motor neurons, also generated conflicting results, making it unclear whether AMPK activity is abnormally activated or inhibited in disease models[168] and whether this contributes to the pathogenesis of the disease[169-171]. Finally,

the question of AMPK's impact on cognitive function is not restricted to neurodegeneration models as pharmacological activation of AMPK by metformin can rescue memory deficits induced by brain ischemia[172] or traumatic brain injury[173].

Given the evidence that Type 2 Diabetes (T2D), or more broadly metabolic dysfunction, is a risk factor for the development of neurological diseases such as stroke, vascular dementia or Alzheimer's Disease, anti-diabetic drugs such as metformin are being explored as potential therapies for neurodegenerative conditions[174]. Metformin is a medication belonging to the biguanide class[175-177], used to treat diabetes. Metformin lowers hyperglycemia by inhibiting hepatic glucose production, increasing insulin sensitivity and enhancing peripheral glucose uptake in muscle[178]. Even though the mechanism of action of metformin is not fully characterized[179], it has very clear benefits on the risk of metabolic syndrome and T2D[180,181] and is often used to enhance AMPK activity by increasing its Thr-172 phosphorylation. Metformin *per os* crosses the blood brain barrier and activates AMPK in mouse brain[182] and can therefore be used in animal studies to determine its effects on cognitive outputs. A majority of publications choose to study

the impact of metformin in T2D mice models (High Fat Diet or db/db mutants) and show improved behavioral response in protocols such as the Morris Water Maze, which measures spatial memory formation, both after short term and long term metformin supplementation[183-185]. In db/db mutants (Leptin Receptor deficient mice), metformin treatment reduces the accumulation of neuronal $A\beta^{1-42}$ and phosphorylated tau (p-tau) in the brain[186], potentially by decreasing $A\beta^{1-42}$ influx and rescuing $A\beta^{1-42}$ efflux through the blood brain barrier[185]. Similarly, metformin can reduce cellular stress markers induced by $A\beta^{1-42}$ in human neuronal stem cells[187]. In a Type 1 Diabetes (T1D) model (induced by streptozotocin injections), metformin treatment only slightly improves the mice response in the T-maze test, however it normalizes a number of neuronal stress markers such as FOX-1 and NeuN (neuronal survival markers) or IL-1beta (inflammation marker)[188,189]. To further confirm the beneficial role of metformin in the context of neurodegenerative diseases, evidence shows that metformin may preserve against dopaminergic neurons degeneration in a MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) induced Parkinson's Disease mouse model[190] and protect striatal cells from mutant huntingtin protein-induced toxicity and mitochondrial dysfunction via AMPK activation[182]. Scopolamine, a muscarinic antagonist, induces dysregulation

of the cholinergic neuronal pathway and memory circuits in the brain, impairing memory, thus simulating Alzheimer's Disease cholinergic deficits[191]. *Mostafa et al*. used this model to test the effect of metformin on cognitive function in a rat model of memory deficit independently of insulin dysfunction. Metformin treatment was associated with improved response in spatial memory tests as well as a reduction of inflammation in the hippocampus and cortex[192]. Supporting the idea that metformin might have beneficial effects on cognitive functions more broadly, metformin treatment can rescue memory deficits induced by injections of pentylenetetrazole (epilepsy mice model)[193], chronic L-methionine administration (model of oxidative stress in the brain)[194] or transient forebrain global ischemia[195]. In this latter model, the authors demonstrated that metformin required AMPK to improve the behavioral response. Despite these positive observations, metformin administration does not always improve cognitive functions, whether in a db/db[186] or WT background[183,193,196]. Metformin is able to penetrate the brain and activate AMPK, however the downstream mechanisms required for its effects on memory formation remain unknown. *Wang et al.* were the first to show that metformin can improve spatial memory formation via enhanced neurogenesis[197]. The authors show that metformin treatment induces neurogenesis through the

activation of the atypical PKC-CBP pathway - a pathway already known for its role in neural precursors differentiation[198] - both in rodent and human cell cultures, and show that this neurogenesis is required for metformin-induced beneficial effects on neural functions. Interestingly, in the liver, the aPKC-CBP pathway is downstream of the metformin-activated kinase, AMPK[197,199].

Finally, these results have translated to humans as several studies now point to the potential beneficial effect of metformin on cognitive performance. In a clinical study including patients suffering from depression and T2D, metformin improved cognitive performance and the depressive symptoms[200]. More recently, results of a pilot clinical trial for metformin in treating mild cognitive impairment (NCT00620191) suggest a beneficial effect of metformin treatment in a cohort of overweight or obese patients, aged over 55 years old, suffering from amnestic mild cognitive impairment (AMCI). These results have to be interpreted with caution due to the small sample size, however they justify the conduct of a larger clinical trial to fully assess the beneficial role of metformin in cognitive disorders[201]. Likewise, a longitudinal cohort study including 1814 individuals - 211 diabetic patients - aged 65-69 years old, (PATH Through Life Study in Australia)

suggests that metformin can provide some level of protection against cognitive impairment and more specifically in regards of verbal learning, working memory and executive function[202]. Not all results are positive though and the effects of long term usage of metformin should be described as a case-control study suggests a potentially increased risk of developing Alzheimer's Disease after long term usage of metformin in a diabetic population[203]. Another clinical study in patients aged over 50 years old showed an alteration of cognitive performance in patients with T2D compared to healthy patients. Metformin seemed to further impair the cognitive functions as assessed by the MMSE (Mini Mental State Examination) score. However, this study fails to properly control for the severity of T2D and metformin intake for each patient[204].

Thesis overview

In order to study the metabolic role of AMPK in the context of neurodegenerative disease, we sought a model organism which could recapitulate age related phenotypes and especially age related neuronal function decline, where human neurodegenerative diseases could be modelled and with available genetic tools.

1.3. *C. elegans* as a model organism

1.3.1. C. elegans as an aging model

C. elegans is a ~1mm soil, free-living nematode found all over the globe that primarily feeds on bacteria such as *E. Coli*. They have a very short reproductive cycle of 3 days and are able to lay several hundred eggs per individual, which is an advantage in lab settings where large synchronized populations can be generated very quickly[205]. *C. elegans* has a lifespan of about 3 weeks and recapitulates a number of age related phenotypes during that time which explains why it became a major model system in the aging research field. These nematodes are usually found as hermaphrodites but males can also be generated and exploited for genetic crosses. Although *C. elegans* has a rather simple morphology and is made up of only 959 somatic cells, it displays specialized tissues such as cuticle, muscle tissue, neuronal tissue or intestinal tissue serving as a metabolic organ (wormatlas.org). The worm's compact genome has been mapped and sequenced which led to the generation by various research labs of thousands of publicly available genetic mutants as well as a genome wide RNAi library (Ahringer Library). More recently, the CRIPSR-Cas9 system has expanded the possibilities of genetic manipulations in all fields and has been very

effectively adapted to *C. elegans* use[206]. Besides these tools, *C. elegans* genome bears between 40% and 70% homology with the human genome, making it a relevant tool for basic research. Finally, *C. elegans* is a transparent organism and allows for *in vivo* imaging of any fluorescently labelled protein in the animal, a major advantage comparing to mouse studies. Finally, given that this model organism can recapitulate key features of aging or neurodegenerative disease and is readily amenable to small molecule screening studies, it can be effectively used for high throughput drug screens.

1.3.2. C. elegans learning and memory / nervous system

Over the past few decades, *C. elegans* has been widely used as a neuroscience model as well a powerful model system to uncover novel mechanisms underlying neurodegenerative processes. Malleable model systems such as *C. elegans* are much needed in a field where few efficient curative therapies exist due to the insufficient characterization of cellular pathways contributing to pathogenesis. The adult hermaphrodite *C. elegans* nervous system is comprised of 302 neurons belonging to two distinct groups: a large somatic nervous system (282 neurons) and a small pharyngeal nervous system (20 neurons)[207-210]. As depicted in Figure **1.4**, the cell bodies

of most neurons are located in the nerve ring or the various ganglia in the head, body, tail of the worm. Despite the widespread prejudice (even among scientists!) that nematodes are not capable of complex behaviors, *C. elegans* actually display a variety of elaborate behaviors, ranging from basic locomotion, foraging or feeding[211] to discrimination between different chemicals, odorants, temperatures or other external stimuli which can lead to a modification of their overall behavior[212-214].

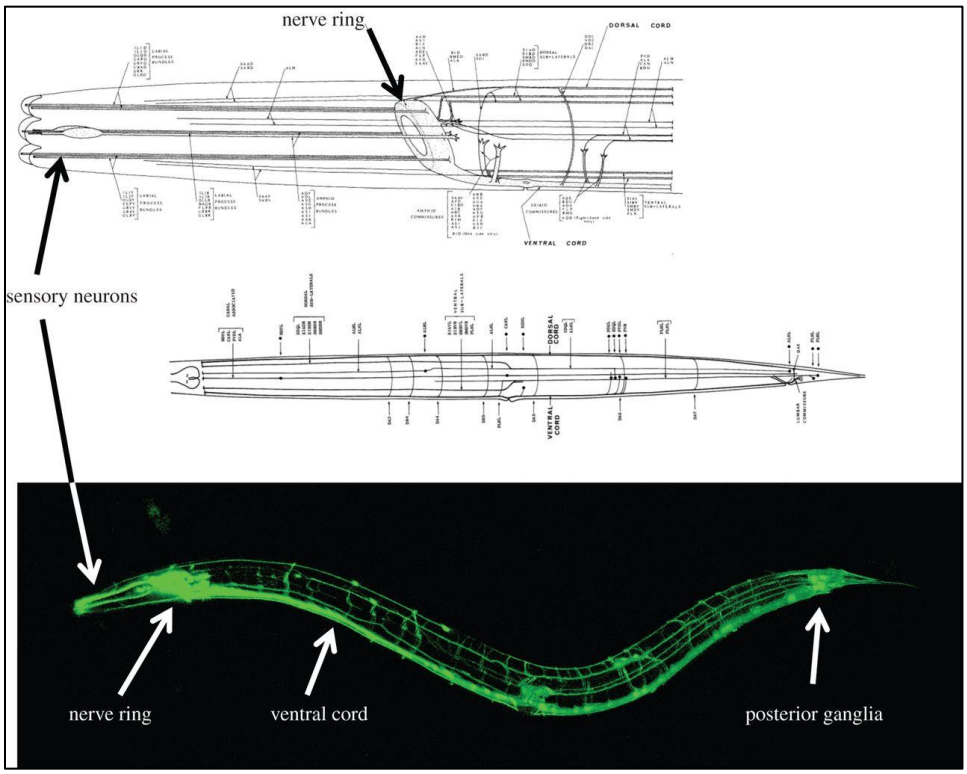

Figure 1.4: *C. elegans* nervous system highlighted by pan neuronal GFP expression[215]

Each of the sensory system in *C. elegans* (mechanosensation, chemosensation, and thermosensation) can be involved in forms of learning and memory and lead to plasticity in the behavior as reported in adaptation, habituation or associative learning assays[216]. Besides the advantages of using *C. elegans* as an aging model, the nematode has a nervous system easily accessible to genetic manipulation or *in vivo* imaging and bears striking similarities with vertebrate neurons. Indeed, *C. elegans* uses the same major neurotransmitters, acetylcholine, glutamate, γ-aminobutyric acid (GABA), serotonin, and dopamine and its vesicular transporters and ion channels function in a similar fashion as in vertebrates[217,218]. Furthermore, the location of chemical synapses is 75% reproducible between individuals thus decreasing variability when experimenting on large number of worms (www.wormatlas.org). Since Kandel and colleagues' work on the marine mollusk *Aplysia*[219], the biological basis of learning has been proven to be highly conserved between invertebrates and mammals[220,221]. Learning and memory behavior in *C. elegans* has been categorized in 3 groups: habituation – or non-associative learning- whereby the worm's initial response to a stimulus progressively declines as it is exposed to the stimulus repeatedly, associative learning which involves the ability learn and remember relevant environmental stimuli and

imprinting, a process taking place during larval development[222]. *C. elegans* has been used as a model to study a number of human neurodegenerative diseases such as: Alzheimer's Disease (AD), Huntington's disease (HD), Amyotrophic lateral sclerosis (ALS), Parkinson's disease (PD), tauopathy or other forms of protein aggregation[223]. These models constitutively overexpress either the wild type, mutated or truncated forms of the human proteins involved in the neurodegenerative disease they model. Depending on the strains, their expression is driven either by a body wall muscle promoter or a neuronal promoter. In case of body wall muscle promoter (eg: *unc-54p*), protein aggregation as well as dysfunctional motility or progressive paralysis can be assayed[224-227]. More interestingly, in case of neuronal expression, protein can be expressed in all neurons (eg: *rab-3p, unc51p, snb-1p, aex-3p*)[228-234] or in a specific subset of neurons. By expressing the protein of interest in specific neurons such as mechanosensory neurons, motor neurons (*acr-2p, mec-3p, mec-7p, unc-30p*) or sensory neurons (*osm-10p*), targeted functional assays can be performed, in this case, scoring worms for their response to mechanic stimuli[235-238]. Likewise, taking advantage of the fact that the *C. elegans* cell lineage is fully characterized, the protein of interest can be driven by promoters which are expressed in a very limited number of neurons such as *dat-1p*

(dopaminergic neurons) or *eat-4p* (glutamatergic neurons), which allows for precise scoring of neuronal loss and characterization of neurodegenerative processes such as abnormal neuron morphology and axonal defects[237,239]. Genetic screens can then be performed on different worm models to identify conserved genes modifying disease progression with the ultimate goal of targeting the pathways uncovered for therapeutic purposes.

The failure in human clinical trials of therapeutic approaches targeting primarily the aggregation and protein misfolding aspect of neurodegenerative diseases such as Alzheimer's Disease has prompted the scientific community to search for novel targets. Evidence from the literature points towards dysregulation of cellular metabolism as a potential causal factor in these brain diseases. Dietary Restriction, a potent intervention for lifespan extension, can also delay the onset of a number of age related disorders, potentially through its effect on multiple metabolic pathways. Unfortunately, dietary restriction does not seem like a viable therapeutic option given its detrimental side effects and compliance difficulty. I therefore focused on one downstream mediator of DR, AMPK, which not only extends lifespan in *C. elegans* and *Drosophila* but also seems to have a prominent role in the mammalian brain. In my thesis work, I investigated the role of AMPK in the nervous system in *C. elegans* in the context of aging and age-related disorders such as Alzheimer's Disease. Given the contradictory evidence from the literature, I first asked whether activation of AMPK, though beneficial for longevity, was beneficial or detrimental in a *C. elegans* model of age-induced neuronal dysfunction. I then sought to understand which of AMPK's multiple downstream targets potentially mediated its effects in neurons.

2. Chapter II Materials and Methods

2.1. *C. elegans* husbandry

2.1.1. C. elegans culture

C. elegans was grown in monoxenic conditions in the laboratory using the *E. coli* strain OP50 as a food source. OP50 is a uracil auxotroph whose growth is limited on standard nematode growth media (NGM) plates. We used the streptomycin resistant variant OP50-1. *E. coli* bacteria were cultured overnight in LB (Lysogeny Broth) at 37 °C, after which 100µl of liquid culture was seeded on plates (6 cm diameter) to grow for 48h at room temperature (RT). Worm stocks were kept in 20ºC incubators unless otherwise noted. For large worm populations, stocks were transferred onto 10 cm diameter NGM plates. After a number of days, worms will completely deplete the bacterial lawn and starve unless transferred to a freshly seeded plate. One method to transfer worms to a new plate is called “chunking”. A sterilized spatula is used to move a chunk of agar from the old plate to a new plate, often resulting in the transfer of several hundred worms. The primary method to transfer worms is to pick single individuals with a worm pick made of platinum wire. The

advantage of platinum wires is that they can heat and cool quickly, allowing them to be flamed (Bunsen burner) between transfers to avoid contamination.

2.1.2. Freezing/thawing strains

C. elegans can be frozen and stored indefinitely in liquid nitrogen (-196ºC) making it an ideal model organism. Freshly starved young larvae (L1-L2 stage) survive the freezing process best while adults, eggs and dauers do not survive well. Large populations of worms were prepared on several large NGM plates (10 cm diameter) seeded with *E. coli* OP50-1. Worms were frozen when the bacterial lawn was exhausted and there was an abundance of L1-L2 larvae. To perform a successful freeze, 15% final volume of glycerol was used in the freezing media and worms were gradually cooled down to -80ºC. Two freezing solutions were used: Liquid Freezing media for long term storage in liquid nitrogen and Soft Agar Freezing media for working stocks of *C. elegans* at -80ºC. The Soft Agar Freezing media has the advantage of keeping the worms suspended thus allowing the worms to be thawed multiple times by scooping a small piece of frozen media. After addition of the freezing media to the larvae, the tubes were placed in a CoolCell cryopreservation freezing container at -80ºC to achieve a 1ºC decrease in temperature per minute. The recovery of

C. elegans from stocks stored in liquid nitrogen is in the range of 35-40% of the total number of animals frozen. All mutants and transgenic strains were frozen even though the viability may vary among them.

2.1.3. Media preparation

Standard Nematode Growth Media plates[240] were prepared in an automated system combining a Mediaclave and a MediaJet. NGM was prepared by adding milliQ H_2O with Agar (2% w/v), BactoPeptone (0.25% w/v) and NaCl (50mM) and autoclaved at 120ºC for 1h before cooling down to 50ºC. After autoclaving, Potassium Phosphate buffer pH6 (25mM), $CaCl_2$ (1mM), $MgSO_4$ (1mM) and Cholesterol (5µg/mL) were added. Drugs such as the antibiotic carbenicilin or phenformin could be added to the solution at this stage. The media was automatically poured in a sterile manner in 6cm (10mL media) or 10cm (25mL media) plates and left to solidify and dry for 24h at RT. Plates were stored in closed plastic containers at 4ºC and used within 1 month of pouring.

2.1.4. RNAi

RNA interference (RNAi) was discovered by Fire and Mello in 1998 and refers to the specific degradation of an mRNA after injection in the worms of the corresponding dsRNA[241]. It was later discovered that feeding worms with bacteria engineered to produce dsRNA could also induce a robust knockdown in gene expression[242]. This method is now routinely used in *C. elegans* labs to perform reverse genetics. The RNAi feeding library used in our lab was the Ahringer Library which contains 16,757 clones, made by cloning gene-specific genomic fragments between two inverted T7 promoters[243,244]. These RNAi clones are expressed by the *E. coli* bacterial strain HT115, which has IPTG (**I**so**P**ropyl-β-D-**T**hio**G**alactoside) inducible T7 polymerase and a disruption of the RNAse III gene (a dsRNAse)[245]. We first streaked individual bacterial strains from the frozen glycerol stock on LB plates containing 100µg/mL carbenicillin antibiotic. We let the plates grow at 37ºC overnight and sent 2-4 individual colonies for sequencing to verify the dsRNA expressed. If the bacterial strain was correct, we grew cultures in LB medium containing 100µg/mL carbenicillin and 12.5µg/mL tetracycline overnight at 37ºC. The bacteria was then seeded on NGM plates containing 100µg/mL carbenicillin and grown at RT for 48h. Prior to

transferring the worms on the RNAi plates (3-4hours), 100µL of 0.1M IPTG was added on top of the bacterial lawn to induce dsRNA expression.

2.2. Strains

2.2.1. C. elegans trains

Some *C. elegans* strains were obtained from the Caenorhabditis Genetic Center, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440). All the other strains were either generated in our lab or generously shared by other labs.

2.2.2. Extrachromosomal arrays generation

Microinjection is an effective method to rapidly generate transgenic animals over-expressing the gene of interest. When making transgenic animals we usually co-transformed the DNA with a visible marker which allowed us to verify the efficiency of the microinjections and to select the transgenic animals. Such markers can include the roller marker (*rol6(su1006*), a mutant collagen) which induces a “roller” phenotype where worms crawl in circles or a fluorescent marker (GFP, RFP, tdTomato) which is visible under a dissecting microscope. In the lab, we typically injected a

DNA solution containing the plasmid carrying the gene/construct of interest and one co-injection marker diluted in H_2O at a final total concentration of 50-100ng/µL of DNA. DNA was injected into the worms with a glass microinjection needle filled with 1µL of DNA solution. A single day 1 adult, well-fed worm was mounted on 2% agarose pads in injection oil (Halocarbon oil), which prevents the worm from desiccating too quickly. The optimal anatomical location to inject the DNA solution is in the cytoplasmic core of the distal gonad (**Fig2.1**). In this anatomical region, the DNA is in contact with multiple germ cell nuclei, thus leading to multiple progeny carrying the transgene. Only one gonad per worm was injected and in order to increase chances of success, 10-15 worms were injected with the same DNA solution.

Figure 2.1: *C. elegans* distal gonad, the optimal location for DNA microinjection[246]

Injected worms were then recovered and transferred to seeded NGM plates in a drop of M9 buffer to allow them to swim out of the oil. The worms were kept at 15ºC overnight to slow down their

physiology and the egg laying rate to allow them to recover. The worms were then kept at 20ºC and the plates scored for the presence of progeny after 2-3 days[247,248].

2.2.3. Genotyping

In order to determine the genotype of a worm, we performed single worm PCR. Because *C. elegans* are hermaphrodite animals, determining the genotype of the parental worm enables us to determine the genotype/s of the progeny. We first lysed a single worm in a 0.2mL microcentrifuge tube containing 5µL of Single Worm Lysis Buffer supplemented with Proteinase K (30mM Tris pH8.0, 8mM EDTA pH8.0, 100mM NaCl, 0.7% IGEPAL CA-630, 0.7% Tween-20 in H_2O with 100µg/mL Proteinase K). The worm was transferred in a sterile manner without bacteria. To lyse the worm and release the genomic DNA, the tube was put in the thermocycler for the following cycle:

Step1) 60ºC for 60min

Step2) 95ºC for 15min

Step3) 4ºC hold

After the lysis reaction was complete, the lysate could be used immediately or stored at -20ºC. We then set up the PCR reaction using 1µL of worm lysate as template and a pair of PCR primers specific to the transgene or mutation, according to the following protocol: 1µM of each primer, 10µL 2X Apex RED Taq DNA Polymerase Master Mix, 1µL of worm lysate, milliQ H_2O up to 20µL final volume. The reaction was run in a thermocycler with the following protocol, modified according to each pair of primers:

Step1) 95ºC for 2min

Step2) 95ºC for 30sec

Step3) 5ºC below melting temperature (Tm) of the primers for 30sec

Step4) 72ºC for time determined by size of amplification (1KB/min)

Steps 2-4 are repeated a total of 30 times

Step5) 72ºC for 2-5min

Step6) 4ºC hold

After PCR reaction, the products can be directly loaded on agarose gels (1-2%) for visualization.

2.3. Behavioral assays

2.3.1. Chemotaxis assay

For both behavioral assays used in this work, the chemotaxis assays were performed in a similar fashion and were based on those described by Bargmann and colleagues[249,250] and adapted to match the published methods from Kauffman *et al* and Saeki et *al*. For the butanone-based assay, shortly before the chemotaxis (10-15min), we spotted 1µL of sodium azide (1M NaN_3) on both sides of the chemotaxis plate (10cm NGM plate) as shown in **Fig2.2**. Then, 1µL of 10% butanone was pipetted on the left side of the assay plate and 1µL of ethanol (95%) was pipetted as a control on the right side of the plate (**Fig2.2**). For the NaCl-based assay, an agar plug was excised from a "high salt" plate (100mM NaCl) with a P1000 plastic tip (to ensure that all plugs were the same volume) and placed on the surface of a "salt-free" plate (6cm, 0mM NaCl) on the left side (**Fig2.2**) and left for 4 hours at RT. Due to osmosis, NaCl from the "high salt" agar plug diffuses into the "salt-free" plate and creates a NaCl gradient on the left half of the chemotaxis plate. Shortly before chemotaxis, the "high salt" agar plug was removed and 1µL of sodium azide was spotted in both sides of the plate. For each condition assayed, a total of 5-6 replicate chemotaxis plates were

prepared. After preparation of the chemotaxis plates, approximately 100-200 animals (per chemotaxis plate) were placed equidistant from the 2 spots (**Fig2.2**) and left to move freely at RT for 1 hour. Worms reaching either side of the plate were anesthetized/paralyzed by the sodium azide thus allowing us to score them. The chemotaxis index (CI) was calculated as CI = (Nb worms on left side – Nb worms on the right side) / (Total nb of worms paralyzed). One assay with 5-6 replicates is considered as one experiment and a minimum of 3 independent experiments were performed, represented by "N" in the figure legends.

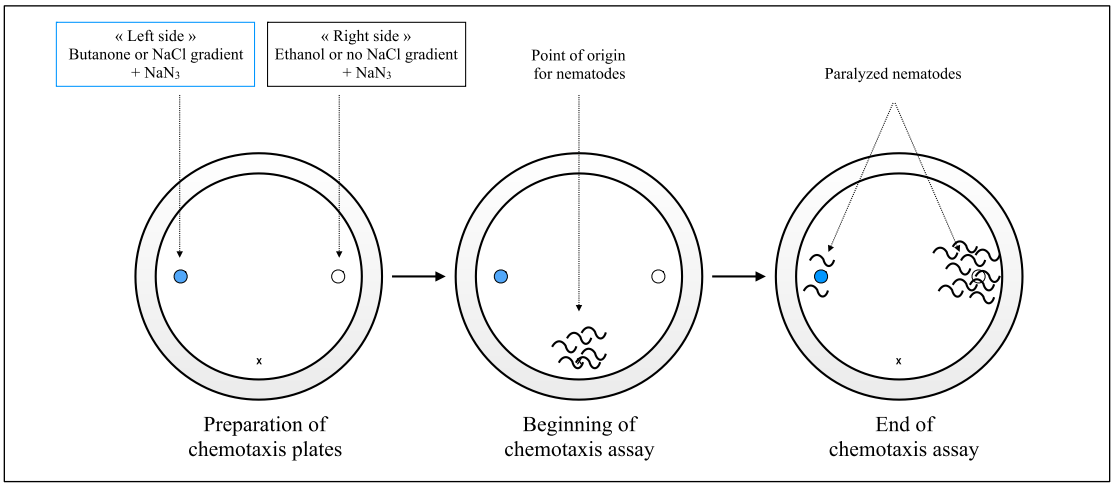

Figure 2.2: Schematic of chemotaxis plates preparation[249,250]

2.3.2. Optimization of the short-term memory assay: pairing of butanone and food

In order to assay neuronal function in *C. elegans* and more specifically behavioral plasticity, we first sought to adapt and optimize in the lab an already published learning and memory assay for *C. elegans* [251,252]. Kauffman *et al*. developed a simple Pavlovian appetitive associative learning and memory assay using the AWC neuron-sensed odorant butanone. As shown in **Fig2.3A**, a population of well-fed synchronized day1 adults was starved in M9 buffer for 1h at RT before being fed in presence of butanone inside the lid (10% diluted in ethanol) and assayed either immediately or after 1-2 hours after re-feeding in absence of butanone. Kauffman *et al* suggest that only the pairing of butanone with *E. Coli* increases chemotaxis to butanone and exposure to food, butanone or starvation separately does not modify the naïve butanone chemotaxis[252]. Their published protocol[251] therefore suggests using naïve worms (non-starved) as the control condition. We first tried to reproduce these published results (**Fig2.3B**) and set up the following controls: naïve worms, worms starved for 1h, worms starved for 1h and conditioned on *E. Coli* only or *E. coli* + ethanol or butanone. Even though we saw a significant increase in attraction towards butanone in the last condition, we also noticed an effect of starvation and mock

conditioning (ethanol conditioning) (**Fig2.3B**) on butanone attraction, suggesting that the increased attraction towards butanone, in our hands, cannot be solely attributed to the associative pairing of butanone and *E. coli*. We next decided to remove the starvation step in the protocol to determine whether butanone could still be paired with the presence of food (**Fig2.3C**). We again saw a slight increase in butanone chemotaxis in the control conditions (Conditioning on *E. coli* +/- ethanol) compared to the naïve worms, however we saw a greater increase in the chemotaxis index in worms trained on butanone. This result suggests that mock conditioned worms (*E. coli* + ethanol) might represent a better control for this assay as simple processing of the worms (washing worms from one plate to another) modifies butanone chemotaxis. The learning index as defined by Kauffman *et al*[252] is the difference between the butanone chemotaxis index (trained worms) and the mock (ethanol) chemotaxis index (control worms). We asked whether we could optimize this protocol and increase the difference in behavior between the control and trained worms by testing various butanone concentrations. Indeed, different butanone concentrations can induce both attractive and aversive behaviors so we aimed to determine the concentration which could both induce the lowest chemotaxis in mock conditioned worms and the highest attraction post training

(**Fig2.3D**). We tested butanone concentrations (diluted in ethanol) ranging from 0.01% to 10% and observed the best response with a 10% concentration (**Fig2.3D**). The 1% and 0.1% concentrations induced a very strong attraction at the basal level, therefore blunting the effect of the butanone associative training. On the other hand, a concentration of 0.01% induced a lower basal chemotaxis (similar to 10% butanone) but could not induce increased attraction after butanone training. Given that 1% butanone can be such a strong attractant at baseline, we asked whether we could use it to set up an aversive associative learning assay by pairing 1% butanone with the negative stimulus starvation (**Fig2.3E**). As expected, we saw a strong chemotaxis towards butanone in our control conditions (naïve worms, starved worms, starved in presence of Ethanol worms). However, we did not see the expected decrease in butanone chemotaxis after starvation in presence of butanone, suggesting that the worms did not associate the negative stimulus which was starvation with the presence of butanone. This could be due to the very strong attraction induced by 1% butanone, which might be hard to reverse with another stimulus. In conclusion, we could not exactly reproduce the published results[251,252](**Fig2.3B**) and even though we managed to optimize the protocol in our hands to observe a positive pairing of butanone with *E. coli* (**Fig2.3C**), the assay

remained very variable, possibly due to the sensitivity of the butanone chemotaxis to starvation (washes can modify the chemotaxis) and the volatile nature of the chemical. Despite lower concentrations of butanone (1%) acting as strong attractant, we could not induce a negative associative learning either. For these reasons, we decided to turn focus towards a different associative learning protocol to assay behavioral plasticity in *C. elegans.*

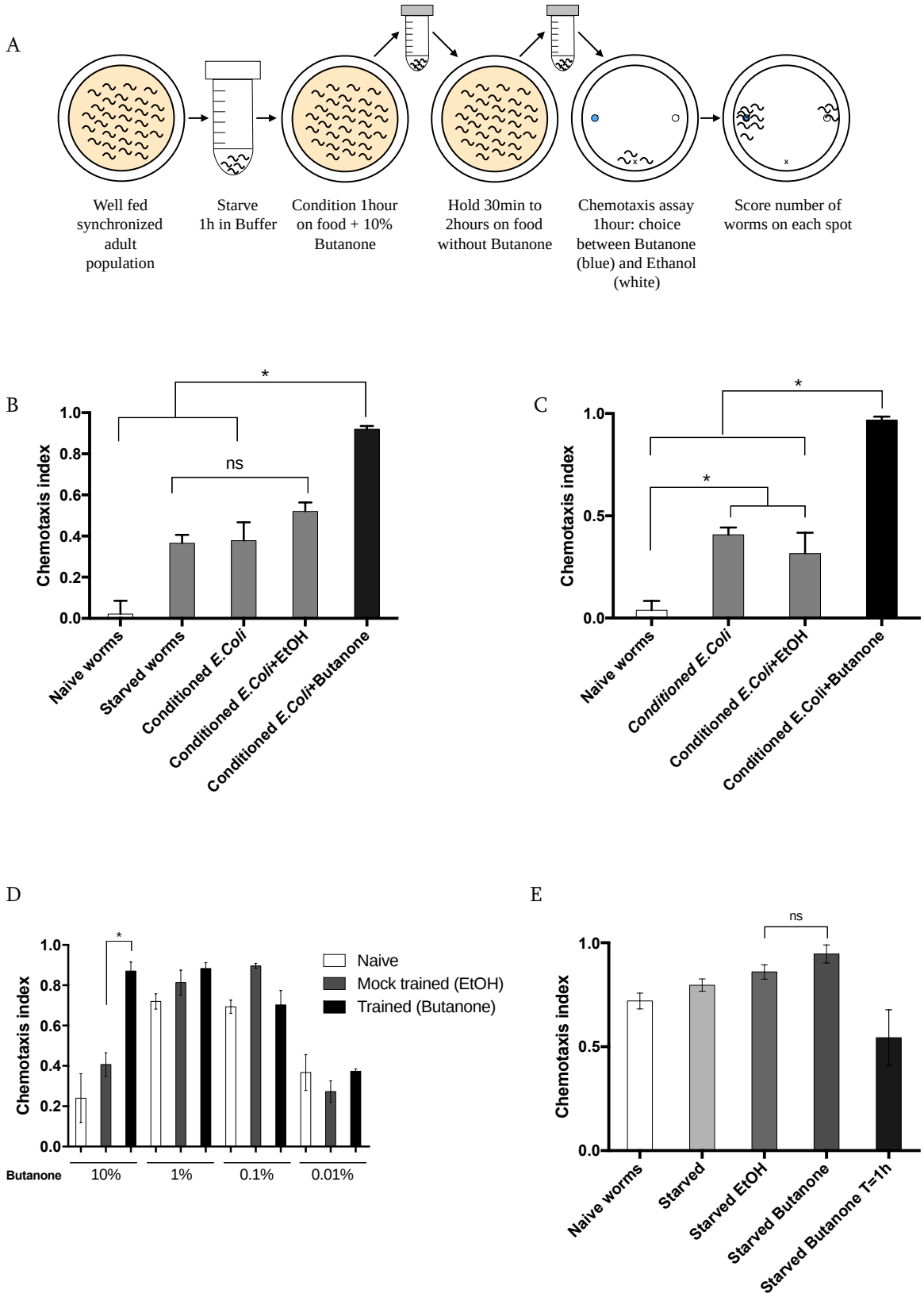

Figure 2.3: Optimization of a short-term memory assay pairing butanone and *E. Coli*

A) Schematic of the short term associative memory assay protocol, adapted from *Kauffman et al.* [253]. All assays in Figure xx are performed with WT N2 animals.

B) Memory assay performed on day1 adult worms. Worms are processed as described and chemotaxis is performed either before conditioning (Naïve worms or Starved worms) or directly after conditioning

C) Worms are not starved prior to conditioning. Worms are conditioned as described and chemotaxis is performed directly

D) Worms are not starved prior to conditioning. Worms are either conditioned on *E.Coli* + EtOH (mock trained) or conditioned on *E.Coli* + Butanone (trained) and chemotaxis is performed directly after. Different concentrations of butanone (0.01% - 10%) are used for conditioning. Chemotaxis is performed in the same manner for all conditions

E) Worms are starved for 1hour on solid plates as described (Starved, Starved EtOH/Butanone 1%) and chemotaxis is performed either directly after or after being transferred to *E.Coli* for 1hour (Starved Butanone T=1h).

2.3.3. Optimization of the NaCl negative association assay

We turned towards a negative associative learning assay[254] to measure behavioral plasticity of the nematodes, which proved to be more robust and more reliable. Similar to the previously protocol described (2.3.1), Saeki *et al* suggest that pairing of NaCl in the media with the negative stimulus starvation can induce a decrease in chemotaxis towards NaCl and that this behavioral change requires the simultaneous presentation of both NaCl and starvation. According to the authors, presentation of either of these stimuli alone does not alter NaCl chemotaxis, thus meeting the criteria for associative learning[254]. The protocol involved conditioning during 4 hours at 20ºC a well-fed synchronized population of nematodes grown on 10cm NGM plates containing ~50mM NaCl and seeded with *E. coli* OP50-1 bacteria. Immediately after conditioning, the worms' chemotaxis was measured (**Fig2.4A**). In order to determine the validity of this assay as an associative learning protocol, we performed the following controls: naïve worms, conditioning on *E. coli*/50mM NaCl, conditioning on *E. coli*/0mM NaCl, conditioning on no *E. coli*/0mM NaCl (**Fig2.4B**). Our training conditioning is no *E. coli*/50mM NaCl. We hypothesized that only the latter conditioning ("trained" worms) would induce a decreased chemotaxis towards NaCl. Indeed,

even though we saw some variation in the NaCl chemotaxis among the control conditions, only the worms exposed to both starvation and NaCl at the same time showed a strong repulsion to NaCl (**Fig2.4B**). Unlike Saeki *et al*, we did not see such a strong attraction to NaCl in the naïve worms (0.6 chemotaxis index in Saeki *et al* versus 0.4 chemotaxis index in our experiment), however, the extent of the behavioral change is very similar (0 chemotaxis index in Saeki *et al* versus -0.7 chemotaxis index in our experiment). This result suggests that nematodes are able to associate NaCl as a negative cue after pairing it with starvation. We also noticed a small drop in NaCl attraction between the naïve condition (chemotaxis index ~0.4) and the control condition no *E. coli*/0mM NaCl indicating that starvation itself might have a small effect on the basal NaCl chemotaxis. In order to control for the effect of starvation alone, we chose to use the no *E. coli*/0mM NaCl condition as our control condition. By looking at the difference between the chemotaxis response in the control condition and the induced NaCl repulsion after training (**Fig2.4C**), we can determine the worms' behavioral plasticity (Learning Index) in this negative associative learning paradigm.

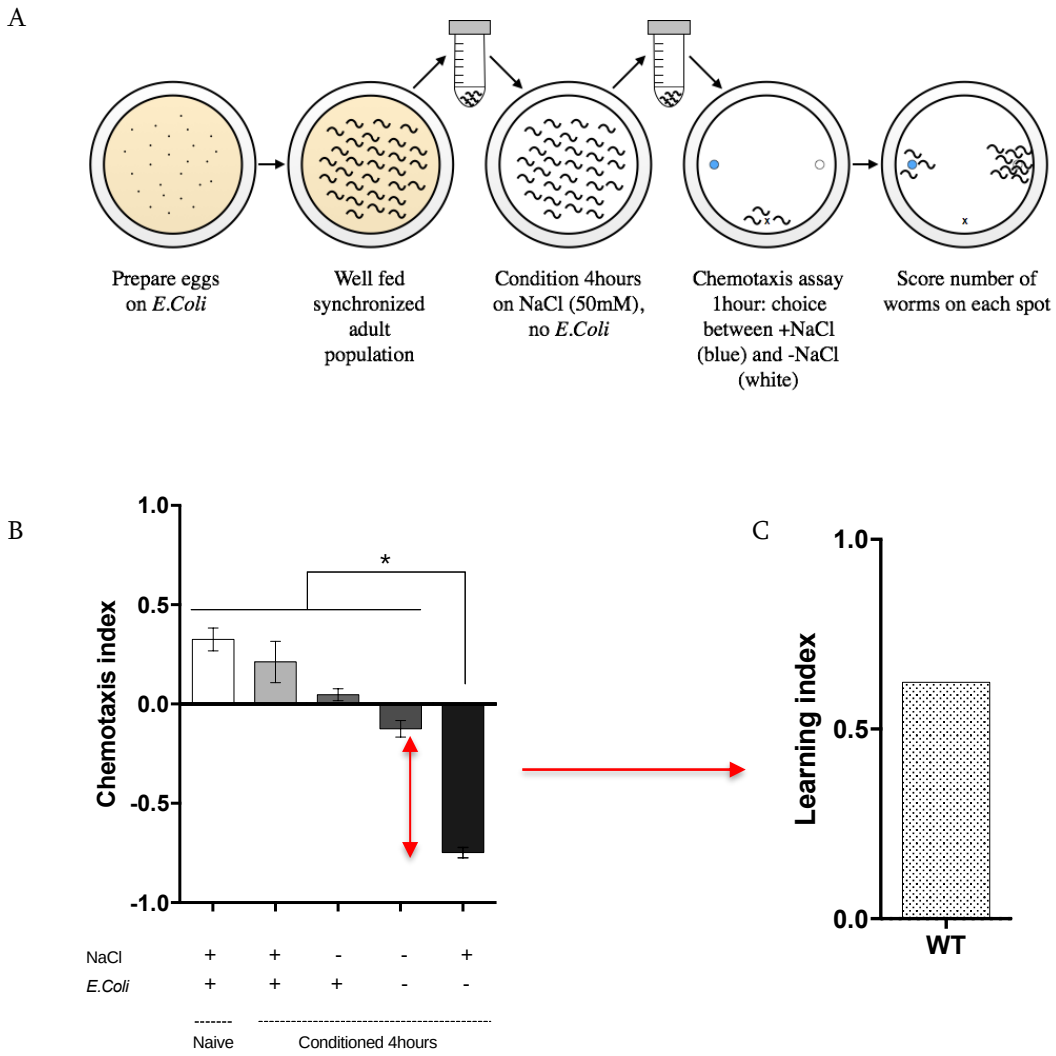

Figure 2.4: Optimization of the short-term NaCl negative association assay

A) Schematic of the short term associative memory assay protocol, adapted from *Kauffman et al.* [253]. All assays in Figure xx are performed with WT N2 animals.

B) Memory assay performed on day1 adult worms. Worms are either unprocessed (Naïve) or conditioned as described. Chemotaxis is performed as previously described.

C) The difference between the Control chemotaxis (-NaCl -*E. Coli*) and the Trained chemotaxis (+NaCl – *E. Coli*) is calculated and plotted as the "Learning Index".

2.4. Lifespans

2.4.1. Synchronization of a C. elegans population

The 'egg laying' method is used when preparing a small, synchronized population of *C. elegans*. We used gravid adults (day1-2 adults) to perform the egg lay. We transferred 6 to 10 gravid adults on a seeded 6cm NGM plate and let the worms lay eggs for 6 hours at 20ºC. After 6 hours, we removed the gravid adult worms from the plate, leaving only the eggs. The 'bleaching' method was used when preparing a large synchronized population of *C. elegans*. In order to obtain a high number of viable eggs, we washed gravid adults from either 2-3 6cm pates or 1-2 10cm plates with M9 buffer and transferred them to a 15mL conical tube. We pelleted the worms by centrifuging for 1min at 2500 rpm at RT and added the following bleach solution: 3mL Bleach 6%, 1.5mL 5N KOH, 5.5mL H_2O. The worms were incubated in the bleach solution for 5min maximum with constant shaking (on a rotator or by manual shaking). Once the worms were fully

dissolved and the eggs were released, the pellet of eggs was washed 3 times in M9 buffer with centrifugation (1min, 2500rpm, RT) between washes to pellet the eggs. After the final wash, the eggs were re-suspended in ~200µL M9 and transferred to a seeded NGM plate.

2.4.2. Lifespan

Lifespans typically started with 100 worms per condition unless otherwise noted. A small egg lay therefore yields enough eggs. For *C. elegans* strains with egg laying defects, embryonic lethality or developmental problems, it is recommended to increase the size of the egg lay or perform a small bleach. At day 1 of adulthood, synchronized worms were transferred to 5 seeded plates (20worms/plate). During the reproductive phase (day1-day7), worms were transferred every day or every other day to avoid contamination by progeny. The worms were typically transferred every other day up to ~day 15, after which they became too old or too fragile to be picked without injury. Plates were freshly seeded for every transfer as described earlier (*2.1.1 C. elegans culture*). In addition to the transfers, worms had to be scored a minimum of every other day. Dead worms were defined by having no response after 3 gentle taps on the head and the tail. Worms which were not alive and fell into one of the following categories were “censored” which removes them from the

statistical analysis: missing, bagged (eggs hatching inside the worm), exploded (usually gonads or intestine coming out of the vulva), dried out on the plate walls, buried into the agar, damaged or killed by the worm pick, contamination of the plate with mold or bacteria. For certain experiments, sterility could be induced pharmacologically using the light sensitive drug 5-fluoro-2'-deoxyuridine (FUDR). We seeded 100µL of 1mg/ml FUDR stock the day before transferring the worms, therefore reaching a final concentration of 10µg/mL of FUDR on a 10mL 6cm NGM plate. After addition of FUDR, the plates were protected from the light by aluminum foil overnight at RT. We transferred day 1 adult animals on FUDR plates and usually left the worms on FUDR until the end of the reproductive phase (day7-day8). FUDR can have lifespan extending/suppressing effects in a genotype specific manner so it is best where possible to avoid using it. As an example, due to an important number of censored subjects, lifespans with the phenformin drug were started with 100-150 worms per condition, with FUDR, to be able to score enough deaths to perform statistical analysis.

2.5. Western blots

2.5.1. Sample collection

When worm populations reached the desired stage, animals were harvested using M9 and transferred to a 15mL conical tube. For each biological sample, approximately 300-500 day1 adults were collected (unless otherwise noted). We centrifuged the worm pellet at 2500rpm for 1 min at RT and washed with M9 buffer 3 times. After the last wash, we aspirated the supernatant, leaving ~500µL of buffer and using a tip rinsed in M9+0.01%Tween-20, the worms were transferred to a 1.5mL eppendorf tube. We pelleted the worms by centrifugation at 500g for 1min at RT and aspirated the remaining buffer and snap froze the pellet in liquid nitrogen. The samples can be stored at -80ºC until further processing. For our experiments, we usually collected 3 biological replicates (same bleach) and repeated the experiment 3 times.

2.5.2. Protein extraction

For each sample, ~75µL of RIPA buffer (50mM Tris pH7.5, 150mM NaCl, 1mM EDTA pH8.0, 1% Triton X-100, 0.1% deoxycholate, 0.1% Sodium Dodecyl Sulfate in H_2O) supplemented with protease and phosphatase inhibitors was added before lysing the worms via sonication in the

Qsonia Q700 sonicator (10secON/10secOFF 3 times with 1 min resting on ice, repeated 4 cycles). The lysate was then centrifuged at 14000g for 5min at 4°C before measuring protein concentration with the BCA method. After determining the protein concentration, we diluted the samples at 1µg/mL in RIPA buffer and added Laemmli buffer supplemented with β-mercaptoethanol to a final 1X concentration. We boiled the samples at 95ºC for 5min and kept the samples at -80ºC until processing.

2.5.3. Protein visualization

We resolved 20µg of total protein by SDS–PAGE using 7.5% acrylamide gels (hand poured) or pre-cast Tris-Glycine gradient (4%-12%) gels. We ran the samples in Tris-Glycine Running Buffer at 80-100V constant for 2 hours or until the running front reached the bottom of the gel. We transferred the proteins to PVDF membranes in chilled Transfer Buffer (1X Transfer Buffer in H_2O and 20% methanol) at 90V constant for 90 min on ice to prevent overheating. After transfer, membranes were stained with Ponceau S to verify the quality of the transfer by incubating the membranes 5 min at RT in Ponceau S. Membranes were then blocked 1h at RT in 5% BSA+TBS-T before incubation with primary antibodies diluted in TBS-T, overnight at 4ºC: phospho-AMPKα

Thr172 (Cell Signaling, 1:1000), β-actin (Abcam, 1:1000). After incubation, the membranes were washed 3 times for 10 min in TBS-T at RT before adding the HRP-conjugated secondary antibody diluted in TBS-T for 1h at RT: anti-mouse (Cell Signaling, 1:2000) and anti-rabbit (Cell Signaling, 1:2000). Bands were visualized by the ECL method using the Gel Doc system (Bio Rad).

2.6. RT-qPCR

2.6.1. Sample collection

When worm populations reached the desired stage, animals were harvested using M9 and transferred to a 15mL conical tube. We centrifuged the worm pellet at 2500rpm for 1 min at RT and washed with M9 buffer 3 times. After the last wash, we aspirated the supernatant, leaving ~500µL of buffer and using a tip rinsed in M9+0.01%Tween-20, the worms were transferred to a 1.5mL eppendorf tube. We pelleted the worms by centrifugation at 500g for 1 min at RT and aspirated the remaining buffer. Under the hood, we added 600µL of Qiazol and snap froze the pellet in liquid nitrogen. The samples can be stored at -80ºC until further processing. For our experiments, we usually collected 3 biological replicates (same bleach) and repeated the experiment twice.

2.6.2. RNA extraction

The first step in extracting the RNA from the collected worms is to crack the cuticle. To do so we used the freeze/thaw method. The samples were thawed in a 37ºC water bath for 2 min, vortexed for 30 sec and frozen in liquid nitrogen for 1 min. This cycle was repeated a minimum of 5 times to ensure successful RNA extraction. Samples were incubated for 5 min at RT before addition of 120µL of chloroform, then vortexed and incubated for 3 min at RT. Following incubation, the samples were centrifuged at 13000rpm for 15 min at 4ºC to separate the aqueous phase. In the hood, we transferred 330µL of the aqueous phase to a new eppendorf tube and mixed with an equal volume of 70% ethanol to precipitate the RNA. The RNA was then extracted on columns using the Qiagen RNeasy Mini Kit and eluted in 30µL RNAse-free H_2O. We determined the RNA concentration by diluting 2µL of RNA in 10mM Tris buffer pH7.5 (1:5 dilution). Pure RNA will show the following ratio in the spectrophotometer (NanoDrop): A_{260}/A_{280} ~1.9-2.1 and A_{260}/A_{230} ~2. Purified RNA can be stored at -80ºC.

2.6.3. Reverse Transcriptase - Quantitative PCR

We prepared cDNA from ~200ng of RNA using the SuperScript VILO MasterMix (ThermoFisher Scientific). We prepared each sample as follows:

200ng RNA

4.5µL H_2O

1.5µL VILO SuperScript MasterMix

For the noRT control, we replaced the VILO SuperScript MasterMix with H_2O. The reverse transcription reaction is run in the thermocycler as follows:

25ºC for 10min

42ºC for 60min

85ºC for 5min

4ºC hold

The cDNA is more stable than RNA and can be kept either at 4ºC or at -20ºC. We set our qPCR in a 96 well plate and measured all our samples in technical triplicates. We used 3 housekeeping

genes available in the lab: *cdc-42*, *pmp-3* or Y45F10D.4. We set up the reactions according to the following protocol:

Primer FORWARD 0.4µL

Primer REVERSE 0.4µL

SYBR green PCR MasterMix 10µL

cDNA (diluted 1:5 in H_2O) 2µL

H_2O 7.2µL

We run the qPCR in the StepOne Plus qPCR machine (Life Technologies) using the SYBR green protocol and analyzing the results with the comparative Ct values method.

2.7. Image acquisition

2.7.1. Neuronal mitochondrial reporter

We injected WT animals with a plasmid expressing under a pan neuronal promoter the fluorescent protein GFP targeted to the mitochondrial outer membrane using the transmembrane domain of TOMM-20 protein. Worms of desired stage were anesthetized in 20X tetramisole diluted in H_2O and mounted on 2% agarose pads on glass slides. After covering the worms with a cover slip, the

slide was sealed with microscopy-grade nail polish in order to avoid desiccation of the sample and sliding of the pad. The samples were imaged using a spinning disc confocal microscope and the image acquisition software NIS-Element. All images were taken with a 100X oil objective using the same exposure time (70ms) with a 488nm wavelength laser. Slices of 0.2µm were taken using the PIEZO technology throughout the whole depth of the worm head. An average of 10 animals were imaged per condition and per time point. After acquisition, all images were analyzed using the raw files with FIJI software. The first 51 slices of the worm head (10.2µm) were extracted, saved as a different file and combined to create a maximum Z-projection to visualize the neuronal mitochondrial morphology. The image was then converted to "temporal-color code" (Rainbow scale), which colors the mitochondrial signal of the Z-projection according to its depth. Images were then blinded and randomized and mitochondrial morphology was qualitatively scored based on 3 categories: fused, wild type and fragmented. These categories were defined based on the aspect of the fission and fusion mutants (*drp-1*$^{-/-}$ and *fzo-1*$^{-/-}$).

2.7.2. Glutamatergic neurons reporter

A reporter strain expressing GFP under a glutamatergic specific promoter (*eat-4*) was anesthetized in 20X tetramisole diluted in H_2O and mounted on 2% agarose pads on glass slides. Worms were imaged using a Zeiss Discovery V8 and Apotome.2-equipped Imager M2 microscope with Axiocam cameras. More specifically, the tail region of the animal was imaged where 5 distinct neurons could be visualized and scored[239]. A minimum of 10 worms were imaged and scored per condition. The results were plotted as the percentage of worms displaying 0,1,2,3,4 or 5 GFP-labeled neurons in the tail.

2.8. Statistical analysis

All experiments were analyzed using Graphpad Prim 7 on Mac. For lifespans and aldicarb assays, *p* values were calculated using the Log-rank (Mantel-Cox) statistical test. Unless otherwise noted in the figure legends, data were plotted as Mean +/- SEM. Statistical analysis was performed using unpaired two tail t-test and *p* values were defined as follows: **ns** no significance, *****<0.05, ******<0.001, *******<0.001 relative to controls.

3. Chapter III AMPK is required to maintain behavioral plasticity with age

In order to investigate the potential effect of AMPK on *C. elegans* nervous system we decided to use an assay which could measure neuronal dysfunction rather than neuronal loss. Even though neuronal death is a popular outcome in ND disease models, neuronal dysfunction induced by toxic peptides like Aβ often precedes actual neuronal loss. We therefore believed that studying age or disease induced neuronal dysfunction might be more relevant to disease progression and could potentially be reversed unlike neurons death.

3.1. Loss of AMPK function mimics age associated and $A\beta^{1-42}$ induced learning deficit

In order to mimic the loss of cognitive abilities observed in the elderly or in AD patients, we decided to assay neuronal function in *C. elegans* and more specifically behavioral plasticity, a negative associative learning assay protocol[254]. As explained in *Materials&Methods*, we optimized this learning assay protocol to reproducibly measure behavioral plasticity in nematodes. Briefly, this protocol is based on the premise that worms are able to modulate their chemotaxis to a neutral element in the media (NaCl) after it has been paired with a deleterious environment (starvation).

In this assay, pairing of NaCl in the media with the negative stimulus starvation can induce a decrease in chemotaxis towards NaCl, only after simultaneous presentation of both NaCl and starvation (**Fig2.4B**). Quantification of this behavioral change therefore reflects neuronal plasticity of the nematodes and can be compared to a primitive learning behavior[254]. In order to determine the validity of this assay as an associative learning protocol, we tested all possible combinations of NaCl and starvation during the training process (**Fig2.4B**). Despite some variation in NaCl chemotaxis among the controls, only worms exposed to both starvation and NaCl simultaneously showed a strong repulsion to NaCl, suggesting that nematodes are able to associate NaCl as a negative cue only after pairing it with starvation (**Fig2.4B**). To control for the effect of starvation alone, we chose to use the no *E. coli*/0mM NaCl condition as our control condition. By comparing the chemotaxis response in the control condition and the induced NaCl repulsion after training (**Fig2.4B**), we can define behavioral plasticity (Learning Index) in this negative associative learning paradigm. Short-term learning has been demonstrated to rely on protein translation whereas long-term memory relies on both gene transcription and protein translation[251]. To validate further the use of this assay as a learning and memory assay, we show that *C. elegans* trained in

presence of a translation inhibitor, Cycloheximide, display an impaired behavioral response (**Fig3.1A**).

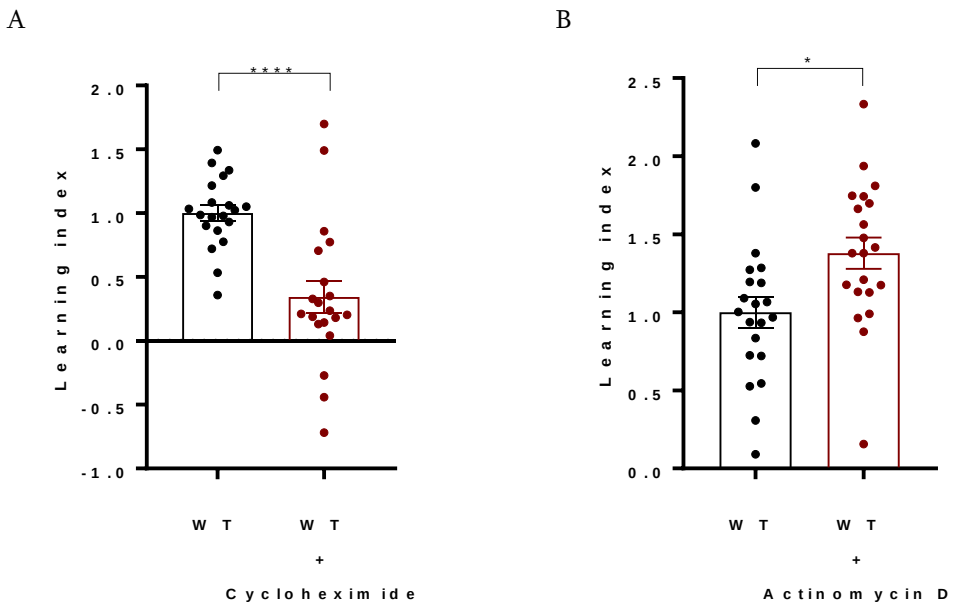

Figure 3.1: Behavioral plasticity in the NaCl based assay requires translation but not transcription

A) Memory assay performed on WT day1 worms untreated or treated with 4h exposure to cycloheximide during conditioning. Means: (WT) 1±0.10, (WT+Cycloheximide) 0.24±0.08. t-test $p<0.0001$. 4 independent biological replicates.

B) Memory assay performed on WT day1 worms untreated or treated with 4h exposure to Actinomycin D during conditioning. Means: (WT) 1±0.10, (WT+ActinomycinD) 0.38±0.10. t-test $p=0.0105$.

Conversely, exposure to the transcription inhibitor Actinomycin D did not impair learning capacity and even significantly improved the behavioral response (**Fig3.1B**) thus meeting the definition of

short term memory. Using this associative learning protocol, we show an age dependent decline in learning ability in *C. elegans* (**Fig3.2**). We observed a significant decline in learning ability in day 3 adults worms (**Fig3.2A**) until an almost complete loss of learning capacity at day 5 of adulthood (**Fig3.2B**), suggesting that this protocol can be useful to study age related changes in neuronal functions.

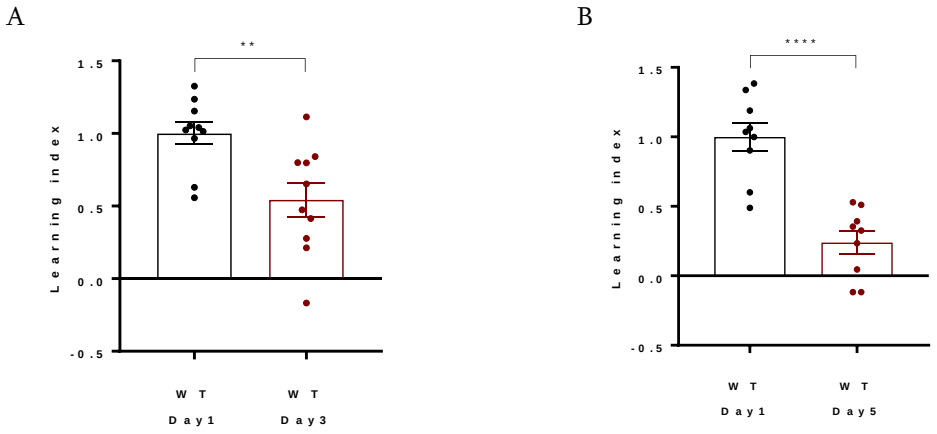

Figure 3.2: Age-dependent loss of behavioral plasticity in WT *C. elegans*

A) Memory assay performed on WT day1 worms versus WT day3 worms. Worms were prepared as described in *Materials&Methods*. Means: (WT day1) 1±0.08, (WT day3) 0.54±0.12. t-test p=0.0044. 2 independent biological replicates.

B) Memory assay performed on WT day1 worms versus WT day5 worms. Worms were prepared as described in *Materials&Methods*. Means: (WT day1) 1±0.10, (WT day5) 0.24±0.08. t-test p<0.0001. 2 independent biological replicates.

We initially hypothesized that the decline in cognitive function observed in *C. elegans* with age is due to a loss of metabolic homeostasis. We therefore asked whether loss of function of the key energetic regulator AMPK, by mutation of one of its catalytic subunits *aak-2*, in *C. elegans*, could impair learning capacity in young animals. We saw no significant difference in the learning index of the *aak-2*$^{-/-}$ mutants compared to WT animals at day 1 of adulthood (**Fig3.3A**). This result is not due to a compensation mechanism, as we obtained similar results by deletion of the other catalytic subunit *aak-1* or deletion of both *aak-1* and *aak-2* (**Fig3.3B,C**). However, by day 4 of adulthood we observed a significant loss of learning ability caused by *aak-2* loss of function compared to WT, which were still able to maintain learning ability (**Fig3.3D**). This result suggests that AMPK activity is important to maintain neuronal plasticity with age.

To mimic the age-related decline in learning ability in AD patients we turned towards a *C. elegans* model of AD which overexpresses the human sequence of the toxic amyloid peptide Aβ^{1-42} in all neurons[255]. As expected, we observed a significant decline in learning ability in young day 1 adult worms expressing the amyloid peptide in their neurons, mimicking the decline in cognitive functions seen in AD patients (**Fig3.4A**).

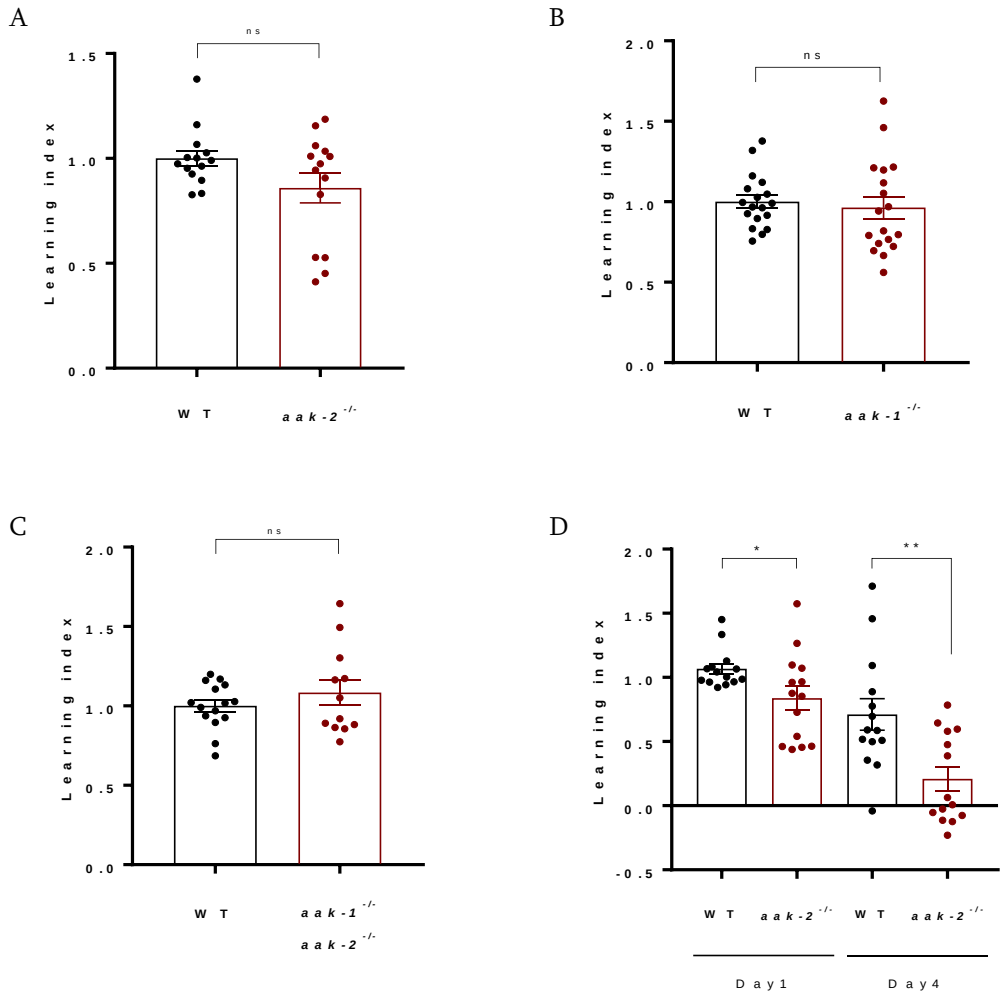

Figure 3.3: Loss of AAK-2 enhances age-related decline in behavioral plasticity

A) Memory assay performed at day1 on WT worms vs. *aak-2*$^{-/-}$ worms. Means: (WT) 1±0.04, (*aak-2*$^{-/-}$) 0.86±0.07. t-test *p*=0.092. 3 independent biological replicates.

B) Memory assay performed at day1 on WT worms vs. *aak-1*$^{-/-}$ worms. Means: (WT) 1±0.14, (*aak-1*$^{-/-}$) 0.96±0.07. t-test *p*=0.6516. 4 independent biological replicates.

C) Memory assay performed at day1 on WT worms vs. *aak-1*$^{-/-}$/ *aak-2*$^{-/-}$ worms. Means: (WT) 1±0.04, (*aak-1*$^{-/-}$/ *aak-2*$^{-/-}$) 1.08±0.08. t-test *p*=0.3178. 3 independent biological replicates.

D) Memory assay performed at day1 and day4 on WT worms vs. *aak-2*$^{-/-}$ worms. Worms were prepared as described in *Materials&Methods*. Means: (WT day1) 1.06±0.04, (*aak-2*$^{-/-}$ day1) 0.84±0.09, (WT day4) 0.71±0.12, (*aak-2*$^{-/-}$ day4) 0.21±0.09. t-test WTday1 vs. *aak-2*$^{-/-}$ day1 *p*=0.0336, t-test WTday4 vs. *aak-2*$^{-/-}$ day4 *p*=0.0031. 3 independent biological replicates.

We chose this particular AD model specifically for its low-level expression of the $A\beta^{1-42}$ peptide. Indeed, the $A\beta^{1-42}$ mRNA is tagged with an "extended" 3'UTR which targets it to degradation by the Non-sense Mediated Decay (NMD) pathway[256]. As a result, the mRNA is targeted to the NMD pathway and degraded, leading to lower levels of protein expression. We confirmed this system by measuring mRNA levels by qPCR after RNAi knockdown of a key component of the NMD complex in *C. elegans*, *smg-1*. As expected, when we disrupt the NMD pathway, we increase the levels of $A\beta^{1-42}$ mRNA produced (**Fig3.4C**). Unlike other published *C. elegans* AD models which display shortened lifespan, this low-level expression strain has a WT lifespan (**Fig3.4D**). We therefore suggest that the effects we are reporting on neuronal plasticity in this AD strain are due to a specific effect of $A\beta^{1-42}$ peptide expression in the neurons rather than overall deterioration of the worms' health.

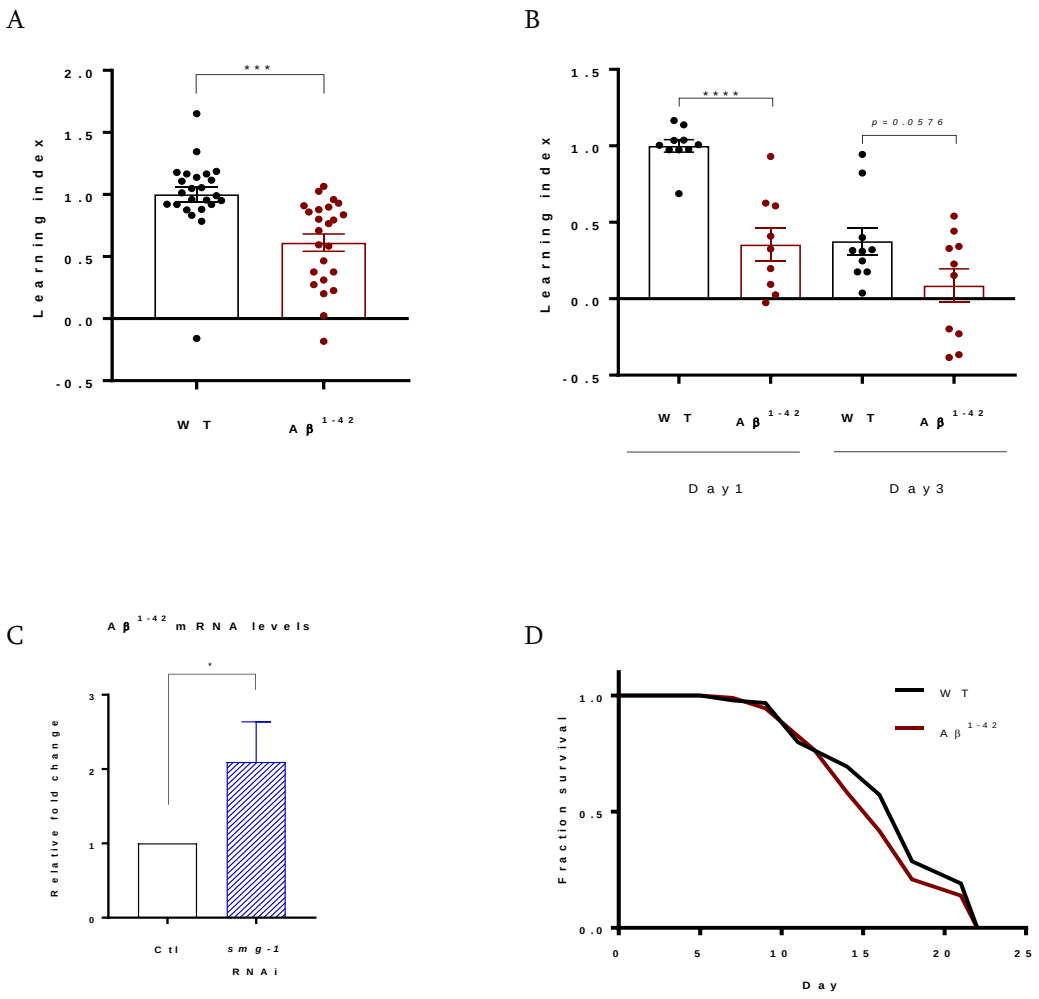

Figure 3.4: Alzheimer's Disease model in *C. elegans* expressing pan neuronal Aβ$^{1-42}$

A) Memory assay performed at day1 on WT worms vs. Aβ^{1-42-} worms. Means: (WT) 1±0.06, (Aβ$^{1-42}$) 0.61±0.07. t-test p=0.0002. 5 independent biological replicates.

B) Memory assay performed at day1 and day3 on WT worms vs. Aβ^{1-42-} worms. Worms were prepared as described in *Materials&Methods*. Means: (WT day1) 1±0.04, (Aβ$^{1-42}$ day1) 0.35±0.11, (WT day3) 0.38±0.09, (Aβ$^{1-42}$ day3) 0.09±0.11. t-test WT day1 vs. Aβ^{1-42-} day1 p<0.0001, t-test WT day3 vs. Aβ^{1-42-} day3 p=0.0576. 2 independent biological replicates.

C) qPCR performed at day1 on WT worms fed HT115 bacteria expressing EV (Ctl) vs. *smg-1* RNAi from hatch. Gene expression is normalized to the housekeeping gene *cdc-42*. Means: (Ctl) 1, (*smg-1* RNAi) 2.10±0.31. t-test p=0.0248. 3 independent biological replicates.

D) Representative lifespan curve of WT vs. Aβ$^{1-42}$ worms fed OP50-1 bacteria. Worms were scored as described in *Materials&Methods*. Medians: (WT) 18 days, (Aβ$^{1-42}$) 16 days. log-rank test p=0.3134.

Here we show that the NaCl based negative association learning assay we chose to use can effectively show age-related and Aβ-induced neuronal dysfunction in *C. elegans*. We further show that *aak-2* loss of function nematodes display an accelerated aging phenotype suggesting a role for AMPK in the maintenance of behavioral plasticity with age. Whether *aak-2* deletion is detrimental in young animals is still unclear. Indeed, even though in **Fig3.3A** we do not measure a significant difference between WT and *aak-2*$^{-/-}$ worms, the latter ones show a significant learning defect at day 1 in **Fig3.3D**. This discrepancy could be explained by the difference in protocols. When testing for behavioral plasticity in older animals, we grow the worms starting from L4 stage in presence of FUDR (5-fluoro-2'-deoxyuridine), a drug which blocks reproduction (cf. *Materials&Methods, 2.4.2 Lifespan*). This allows us to age the worms without having to separate them from their abundant progeny. Furthermore, in order to maintain large worm populations without having to transfer them, which could interfere with the assay, we seed the plates with a slightly thicker bacterial lawn. These differences in protocol could account for the discrepancy we observed when measuring behavioral plasticity in the AAK-2 loss of function mutants. Finally, it could be argued that using starvation, a condition known to activate AMPK, as a negative stimulus in our

conditioning protocol might induce a bias. Even though we cannot completely exclude this possibility, the observation that *aak-2*$^{-/-}$ or *aak-1/2*$^{-/-}$ mutants show a learning capacity similar to WT animals in **Fig3.3A,C** suggests that AMPK activation is not required for the sensing of NaCl or starvation. In order to support this hypothesis, we planned to reproduce these results with another learning and memory paradigm pairing two completely different stimuli (such as food and the volatile chemical butanone) which are sensed by different neuronal circuits.

3.2. AMPK activation improves learning and maintains neuronal function with age

The observation that *aak-2* loss of function worms have an accelerated decline in learning capacity prompted us to analyze an AMPK activation model. It was previously shown that ubiquitous expression of the truncated catalytic subunit of AMPK renders it constitutively active (CA-AAK2) and extends lifespan in *C. elegans*[79].

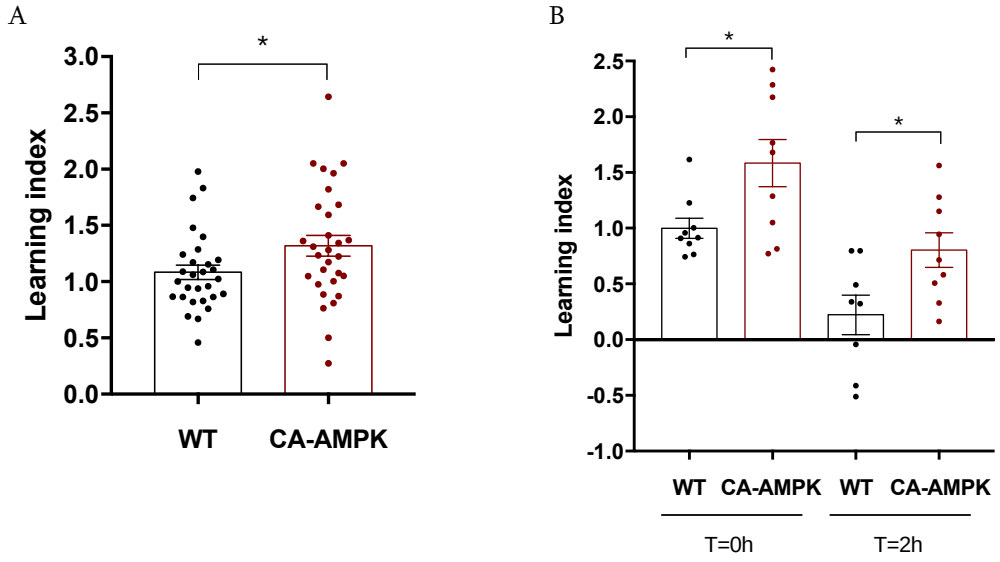

Figure 3.5: Constitutive activation of AMPK improves behavioral plasticity in young worms

A) Memory assay performed at day1 on WT worms vs. CA-AMPK worms. Means: (WT) 1.08±0.06, (CA-AMPK) 1.32±0.09. t-test p=0.00426. 6 independent biological replicates.

B) Memory assay performed at day1 on WT worms vs. CA-AMPK worms, either immediately after conditioning (T=0h) or 2h after refeeding on *E. Coli* (T=2h). Means: (WT T0h) 1±0.09, (CA-AMPK T0h) 1.58±0.21, (WT T2h) 0.22±0.18, (CA-AMPK T2h) 0.80±0.15. t-test WT T0h vs. CA-AMPK T0h p=0.0218, t-test WT T2h vs. CA-AMPK T2h p=0.0255. 2 independent biological replicates.

Interestingly, we observed that CA-AAK2 worms had an increased learning capacity at day 1 of adulthood (**Fig3.5A**), correlating with the overall beneficial effects of AMPK activation on lifespan and the opposite effect measured in the mutant animals. In addition to increased learning capacity immediately after training (T=0), activation of AMPK improves the retention of the negative association after 2 hours of re-feeding on *E. Coli* (T=2h) compared to WT worms (**Fig3.5B**) suggesting a beneficial role for AMPK in both the formation and retention of a basic negative association. Consistent with the *aak-2* loss of function data (**Fig3.3D**), CA-AAK2 worms showed increased learning capacity compared to WT worms at day 5 of adulthood and strikingly do not seem to display any age-related decline when comparing CA-AAK2 worms at day 1 vs. day 5 (**Fig3.6A**). To test whether the beneficial effect of AMPK on age related decline in behavioral plasticity could also rescue $A\beta^{1-42}$-induced neuronal defects, we crossed the AMPK genetic activation model strain CA-AAK2 in our AD model strain. We show that AMPK activation can fully rescue the $A\beta^{1-42}$ induced defects in young worms suggesting that the detrimental effect of a proteotoxic peptide can be alleviated by modulation of a key cellular energy sensor (**Fig3.6B**). We

controlled Aβ$^{1-42}$ mRNA levels by qPCR to verify that transgene expression was not decreased by CA-AMPK overexpression (**Fig3.6C**).

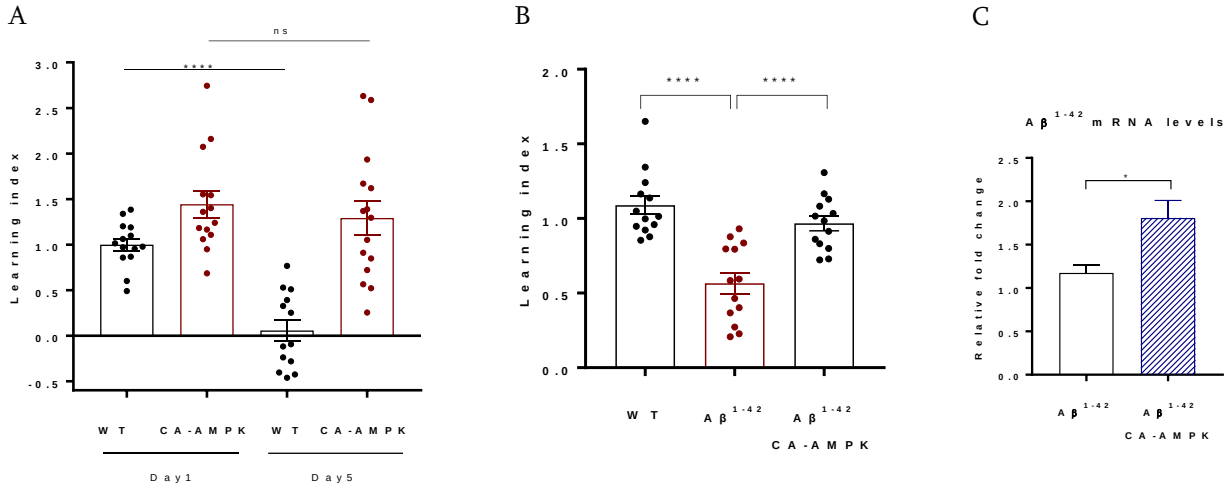

Figure 3.6: Constitutive activation of AMPK rescues age-related and Aβ$^{1-42}$ induced neuronal defects

A) Memory assay performed at day1 and day5 on WT worms vs. CA-AMPK worms. Worms were prepared as described in *Materials&Methods*. Means: (WT day1) 1±0.07, (CA-AMPK day1) 1.44±0.15, (WT day5) 0.06±0.12, (CA-AMPK day5) 1.29±0.18. t-test WTday1 vs. WT day5 $p<0.0001$, t-test CA-AMPK day1 vs. CA-AMPK day5 $p=0.5245$. 3 independent biological replicates.

B) Memory assay performed at day1 on WT vs. Aβ$^{1-42}$ vs. Aβ$^{1-42}$ x CA-AMPK worms. Means: (WT) 1.09±0.06, (Aβ$^{1-42}$) 0.57±0.07, (Aβ$^{1-42}$ x CA-AMPK) 0.97±0.05. t-test WT vs. Aβ$^{1-42}$ $p<0.0001$, t-test WT vs. Aβ$^{1-42}$ x CA-AMPK $p=0.1355$, t-test Aβ$^{1-42}$ vs. Aβ$^{1-42}$ x CA-AMPK $p=0.0001$. 3 independent biological replicates.

C) qPCR performed at day1 on Aβ$^{1-42}$ vs. Aβ$^{1-42}$ x CA-AMPK worms. Gene expression is normalized to the housekeeping gene *cdc-42*. Means: (Aβ$^{1-42}$) 1.17±0.09, (Aβ$^{1-42}$ x CA-AMPK) 1.81±0.20. t-test $p=0.0465$. 3 independent biological replicates.

Given the data from our genetic model, we asked if we could recapitulate these effects with a pharmacological intervention. We chose phenformin, the analog of the widely used metformin drug, for the vast array of publications pointing towards a beneficial role of metformin on the brain (**cf. *Introduction***). Furthermore, phenformin has already been shown, in *C. elegans*, to activate AMPK and extend lifespan in an AMPK dependent manner[257]. We first showed that 4.5mM supplementation of phenformin in the nematodes' culture media could increase AMPK activity as measured by AAK-2 Thr-172 phosphorylation (**Fig3.7A**). We confirmed that with our protocol we could effectively extend lifespan in *C. elegans* in an *aak-2* dependent manner (**Fig3.7B**). Worms grown in presence of phenformin at this concentration displayed an enhanced behavioral response at day 1 of adulthood (**Fig3.7C**). More interestingly, phenformin supplementation was equally able to rescue the $A\beta^{1-42}$ induced neuronal deficits in young animals suggesting that pharmacological targeting of AMPK might produce similar beneficial effects as our genetic model. This highlights the translational potential of AMPK as a therapeutic target.

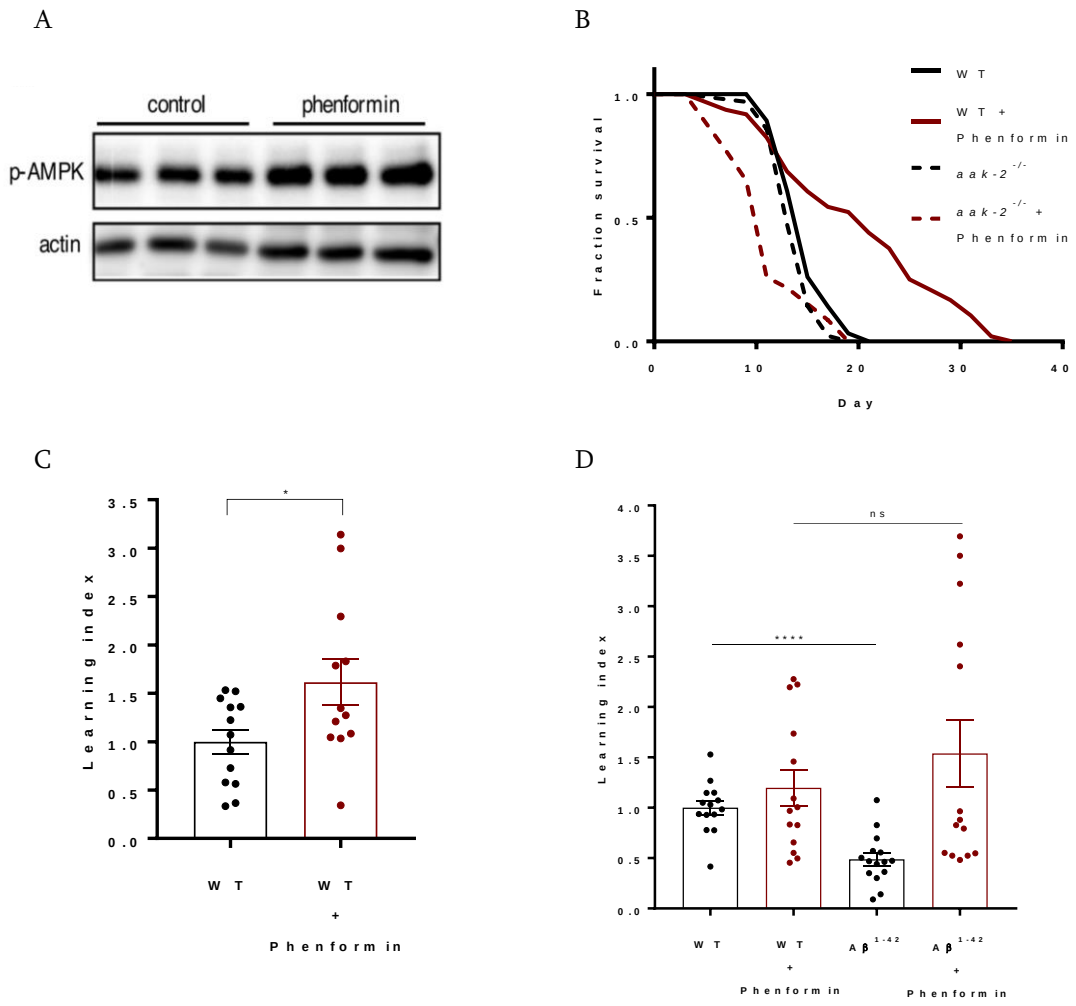

Figure 3.7: Pharmacological activation of AMPK via phenformin recapitulates the genetic activation model results

A) Western blot of p-AMPK levels in day1 WT worms on control media (Ctl) vs. phenformin supplemented media for 24h from L4 stage. 3 independent biological replicates.

B) Representative lifespan curve of WT vs. *aak-2*$^{-/-}$ worms fed OP50-1 bacteria on control vs. phenformin supplemented media started from day1. Worms were scored as described in *Materials&Methods*. Medians: (WT) 15 days, (WT+Phenformin) 21 days, (*aak-2*$^{-/-}$) 15 days, (*aak-2*$^{-/-}$+Phenformin) 10 days. WT vs. WT+Phenformin lifespan extension +40% log-rank test $p<0.0001$, *aak-2*$^{-/-}$ vs. *aak-2*$^{-/-}$+Phenformin lifespan extension -33.3% log-rank test $p=0.0012$.

C) Memory assay performed at day1 on WT worms grown from hatch on control media (WT) vs. phenformin supplemented media (WT+Phenformin). Means: (WT) 1±0.12, (WT+Phenformin) 1.62±0.24. t-test p=0.0293. 3 independent biological replicates.

D) Memory assay performed at day1 on WT vs. $A\beta^{1-42}$ worms grown from hatch on control media vs. phenformin supplemented media. Means: (WT) 1±0.07, (WT+Phenformin) 1.20±0.18, ($A\beta^{1-42}$) 0.49±0.06, ($A\beta^{1-42}$+Phenformin) 1.54±0.33. t-test WT vs. $A\beta^{1-42}$ p<0.0001, t-test WT+Phenformin vs. $A\beta^{1-42}$+Phenformin p=0.3760. 3 independent biological replicates.

Overall, these data are in accordance with previous publications in mammalian systems showing a beneficial role of AMPK in the context of neurodegeneration. Here we correlate the behavioral deficits measured in the *aak-2*$^{-/-}$ mutants and the AD model, suggesting that neuronal expression of $A\beta^{1-42}$ might induce a state of metabolic imbalance or stress which can be rescued by restoring AMPK activity. To further confirm this hypothesis, we should look at the neuronal AMPK activity in our worm AD model and ask if it is decreased. This result would be very interesting as it has been reported in the literature that the toxic amyloid peptide Aβ can cause abnormal upregulation of AMPK potentially contributing to disease pathogenesis[164]. Unfortunately, western blot of tissue specific proteins in *C. elegans* still remains a challenge. With CRISPR technologies developing and providing new tools every day, it should be possible to express a tagged single copy of AAK-2 specifically in the nervous system of the worm. The tag

would allow us to immunoprecipitated selectively the AAK-2 protein expressed in the neurons for further analysis of its phosphorylation status. Here, we also demonstrate that both genetic and pharmacological activation of AMPK leads to a complete rescue of the AD strain impaired response. However, we have not characterized how AMPK activation affects the AD model. Indeed, even though we showed no alteration of the Aβ transcript levels, it will be critical to examine protein levels, by either western blot or thioflavin staining for example. AMPK might enhance its degradation or modulate its aggregation properties, two mechanisms which could explain the improved phenotype. If we were to observe lower levels of total Aβ protein or modification of the aggregation profile, it could point towards autophagy as a downstream mechanism of AMPK. However, if the peptide accumulation is unchanged in the CA-AMPK line it could suggest that AMPK is acting downstream of Aβ toxicity by modulating the cellular response to its accumulation.

4. Chapter IV AMPK requires mitochondrial fusion to improve behavioral plasticity

Mitochondria are essential organelles involved in diverse cellular processes including, but not limited to, ATP production, regulation of apoptosis, calcium homeostasis and reactive oxygen species production. Mitochondria are very dynamic organelles which can either fuse or divide depending on the energetic requirements of the cell. The dynamic capacity of mitochondria is essential for their maintenance, function and cytoplasmic distribution in the cell to ensure its presence at sites requiring increased production of ATP, such as synapses[258]. Mitochondrial morphology directly correlates with mitochondrial function. On one side, mitochondrial fusion allows exchange of mitochondrial content such as proteins of the electron transport chain complexes or mitochondrial DNA (mtDNA) which can protect the cells against degradation or mutation of the mtDNA and maintain efficient oxidative phosphorylation[258-262]. On the other side, fission generates smaller mitochondria which can be eliminated through a process known as mitophagy[263]. Mitochondrial fission and mitophagy therefore enable the cell to isolate and dispose of damaged mitochondria. At the cellular level, mitochondria dynamics play a role in cell proliferation, nutrient utilization and energy metabolism[264,265].

Mitochondrial fission is controlled by the soluble Dynamin-Related Protein1 (DRP1), which is ubiquitously expressed in mammals but more highly expressed in the brain[266]. DRP1 is conserved in all eukaryotes including *C. elegans* (*drp-1*)[258]. DRP1 accumulates on the mitochondrial outer membrane, preferentially at contact sites with the endoplasmic reticulum, to constrict the mitochondrial membrane and induce fission upon GTP (guanosine triphosphate) hydrolysis[267,268]. Mitochondrial fusion is regulated by mitofusins (MFN1 and MFN2) as well as OPA1 (Optic Atrophy 1) protein, which control outer mitochondrial membrane and inner mitochondrial membrane fusion respectfully [258,269,270]. These proteins are also conserved in eukaryotes[271] and *C. elegans* where they are encoded by two genes: *fzo-1* (MFN1/2 homolog) and *eat-3* (OPA1 homolog). Deletion or reduction of DRP1 expression leads to "hyperfusion" of the mitochondrial network characterized by increase in length and interconnectivity of mitochondrial filaments in mammalian cell lines[272]. The same phenotype can be readily observed *in vivo* in a *C. elegans* KO strain (cf data below). DRP1 activity can also be physiologically inhibited, through starvation for example, which can spare mitochondria from degradation through fusion of the network in order to maintain ATP production[273]. On the other side, mitofusins are required for

mitochondrial fusion and their loss of function leads to a fragmented mitochondrial network[274,275] (cf below). Studies in cultured neurons and zebra fish have further demonstrated a role for mitofusins in mitochondrial transport through their interaction with Miro proteins[276,277].

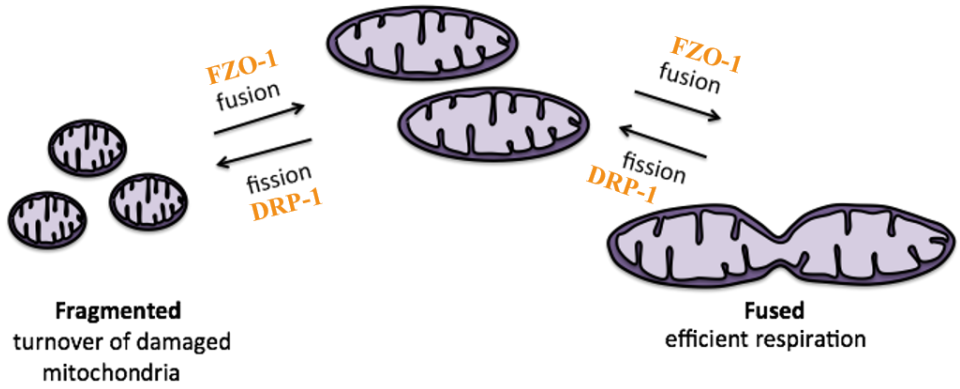

Figure4.1: Illustration of mitochondrial fusion/fission process in *C. elegans*

Neurons are very energy dependent and require a tight regulation of calcium homeostasis for synaptic function thus making them very sensitive to modification in mitochondrial function. Given the importance of mitochondrial function and distribution in neuronal cells, it is not surprising that disruption of mitochondrial dynamics machinery leads to neuronal dysfunction and neurological diseases[278]. Two groups of neuropathies have been linked, in humans, to mutations in *MFN1/2* or *DRP1*: Dominant optic atrophy (DOA) and Charcot Marie Tooth disease (CMT)[279,280]. DOA is characterized by degeneration of the visual nerve leading to visual

impairments including visual loss[279] and 60-80% of patients suffering from DOA display a mutation in the mitochondrial fusion regulator *OPA1*[281] whereas axonal CMT, a motor and sensory neuropathy, is associated with mutation in *MFN2*[282].

Dysregulation of mitochondrial function and mitochondrial dynamics in particular have been associated with neurodegenerative disorders such as AD, HD and PD[35]. In AD, multiple aspects of mitochondrial function has been shown to be altered[35]; AD brains display a deficiency in TCA (tricarboxylic acid or Krebs) cycle enzymes[283], impaired PGC1α expression contributes to AD progression[284], Prenisilin 1 mutation causes mitochondrial axonal transport defects[285], Aβ peptide accumulates on and blocks mitochondrial import channels[286], increased percentage of mtDNA mutations has been described in AD brains[287] and calcium homeostasis is severely altered[288,289]. Furthermore, neuronal expression of mitochondrial dynamics machinery protein is significantly reduced in AD hippocampi[290]. Mitochondrial dynamics are also altered in AD, as earlier studies done in AD neurons described modifications of mitochondrial size and number as well as an increase in mitochondria with broken cristae[287]. Exposure of cultured neurons to

aggregated Aβ peptide or overexpression of APP and APP^{sw} mutant can also lead to mitochondrial fragmentation[290-292]. Alteration of mitochondrial capacity to fuse or divide consequently leads to axonal transport defect and abnormal mitochondrial distribution between the soma and the dendrites[290,293].

Given the relevance of mitochondrial dynamics alterations in AD and it requirement in AMPK mediated longevity[35,135] we decided to further explore the effect of mitochondrial morphology changes in associative learning and the possible downstream requirement of fusion and/or fission for AMPK mediated benefits on behavioral plasticity.

4.1. AMPK requires mitochondrial fusion to promote learning

AMPK regulates mitochondrial biology by enhancing both mitochondrial biogenesis and mitophagy (**cf. *Introduction***) and recent evidence shows that AMPK also regulates mitochondrial morphology[294]. Indeed, there is a direct requirement of mitochondrial dynamics for AMPK mediated lifespan extension in *C. elegans*[294]. Mitochondrial biology dysregulation and especially fragmentation of the network has been causally linked to the pathogenesis of several neurodegenerative diseases[35,258]. We therefore asked whether AMPK mediates its beneficial effects on age-related and $A\beta^{1-42}$ induced loss of neuronal plasticity by maintaining cellular metabolic homeostasis via regulation of the mitochondrial network. To examine whether mitochondrial fragmentation might impact learning and memory in *C. elegans*, we first studied deletion of the mammalian Mfn1/2 homolog in *C. elegans, fzo-1,* and observed a severe deficit in behavioral plasticity (**Fig4.2A**). This mutation prevents mitochondria from fusing together thus leading to a fragmented mitochondrial network[294]. To test whether this defect was caused by deletion of the gene itself or by induction of mitochondrial fragmentation we looked at the double deletion of both *fzo-1* and *drp-1* (**Fig4.2B**).

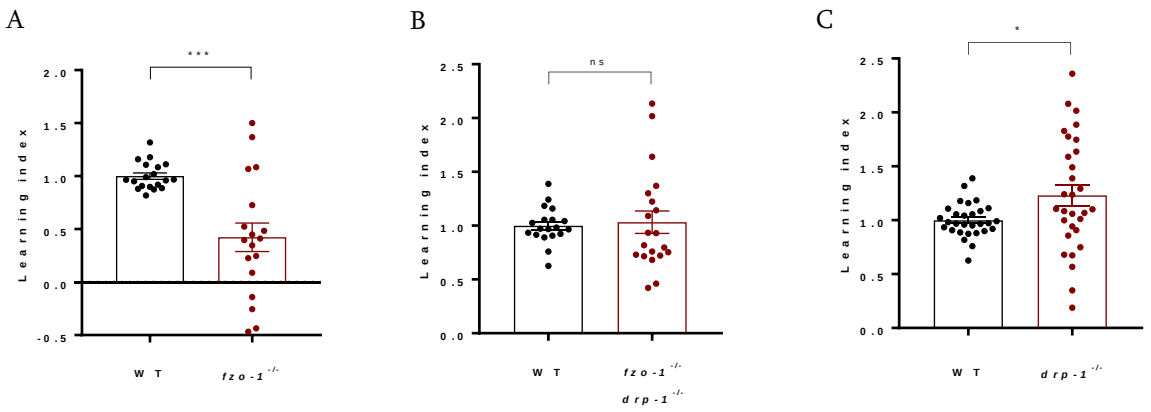

Figure 4.2: A connected mitochondrial network is required for behavioral plasticity

A) Memory assay performed at day1 on WT worms vs. *fzo-1*$^{-/-}$ worms. Means: (WT) 1±0.03, (*fzo-1*$^{-/-}$) 0.42±0.13. t-test *p*=0.0001. 4 independent biological replicates.

B) Memory assay performed at day1 on WT worms vs. *fzo-1*$^{-/-}$x. *drp-1*$^{-/-}$ worms. Means: (WT) 1±0.04, (*fzo-1*$^{-/-}$x. *drp-1*$^{-/-}$) 1.03±0.10. t-test *p*=0.7797. 3 independent biological replicates.

C) Memory assay performed at day1 on WT worms vs. *drp-1*$^{-/-}$ worms. Means: (WT) 1.03±0.03, (*drp-1*$^{-/-}$) 1.23±0.10. t-test *p*=0.0277. 6 independent biological replicates.

Even though this strain is deficient in both fusion and fission, it is still viable and it maintains a connected mitochondrial structure[294]. The double mutation can fully rescue *fzo-1*$^{-/-}$ induced behavioral plasticity deficit, suggesting that worms require a connected mitochondrial network to form associative learning (**Fig4.2B**). Interestingly, induction of a more fused mitochondrial network by deletion of the fission factor *drp-1* alone further enhances learning ability in day 1 adult worms (**Fig4.2C**). We initially hypothesized that mitochondrial dynamics are a mechanism

downstream of AMPK and sought to exclude the possibility that the effect observed in the mitochondrial mutant strains might be due to changes in AMPK activity levels. We performed western blot analysis of Thr-172 phosphorylation levels in the mitochondrial mutants and saw a slight increase in p-AMPK in all strains, more significantly in *drp-1* and *fzo-1* mutants (**Fig4.3**).

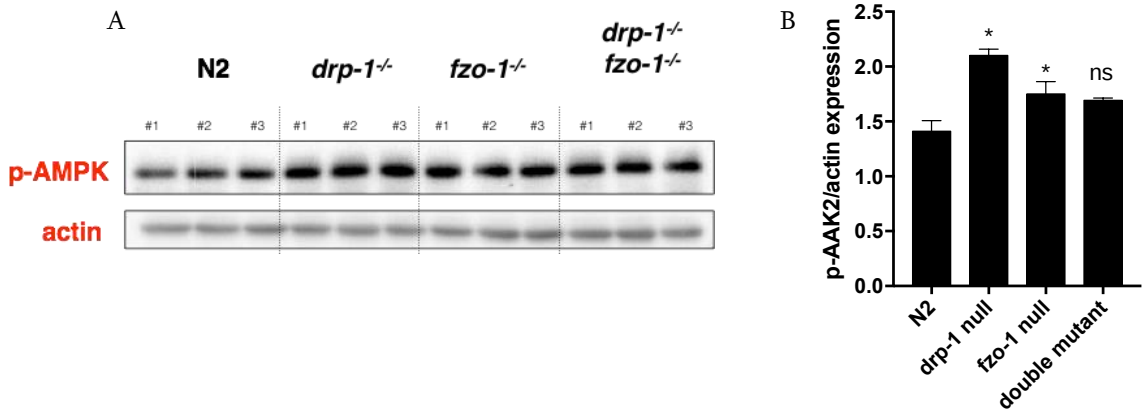

Figure 4.3: p-AMPK levels in mitochondrial mutants

A) Western blot of p-AMPK levels in day1 WT worms vs. mitochondrial mutants *fzo-1*$^{-/-}$, *drp-1*$^{-/-}$ and *fzo-1*$^{-/-}$ *x drp-1*$^{-/-}$. 3 independent biological replicates.

B) Quantification in Image J. p-AMPK levels normalized to actin levels.

These results do not correlate with the behavioral phenotype we observed and it therefore seems unlikely that AMPK acts downstream of *drp-1* and *fzo-1* mutations. Given that *drp-1*$^{-/-}$ mutant shows the highest p-AMPK increase, we could potentially study the behavioral plasticity of *drp-1*$^{-/-}$ in an *aak-2*$^{-/-}$ to completely exclude this possibility.

Given these results, we asked whether mitochondrial fusion capacity was required downstream of AMPK to enhance behavioral plasticity. Deletion of the fusion factor *fzo-1* completely blocked the ability of CA-AMPK to promote associative learning (**Fig4.4A**). This can be entirely rescued by deletion of both *fzo-1* and *drp-1* suggesting that AMPK activation requires a connected mitochondrial network to improve behavioral plasticity (**Fig4.4B**). Promotion of a hyper-fused mitochondrial network shows additive beneficial effects with CA-AMPK on learning (**Fig4.4C**).

We showed that AMPK requires fusion of the mitochondria to enhance neuronal plasticity and therefore hypothesized that directly promoting mitochondrial fusion by deleting *drp-1* might have beneficial effects on the AD model worm. However, *drp-1* mutation did not rescue $A\beta^{1-42}$ induced learning defects (**Fig4.5A**). Induction of mitochondrial fragmentation by *fzo-1* mutation did not display additional learning defect with $A\beta^{1-42}$ expression (**Fig4.5B**) suggesting mitochondria may already be fragmented in the AD strain.

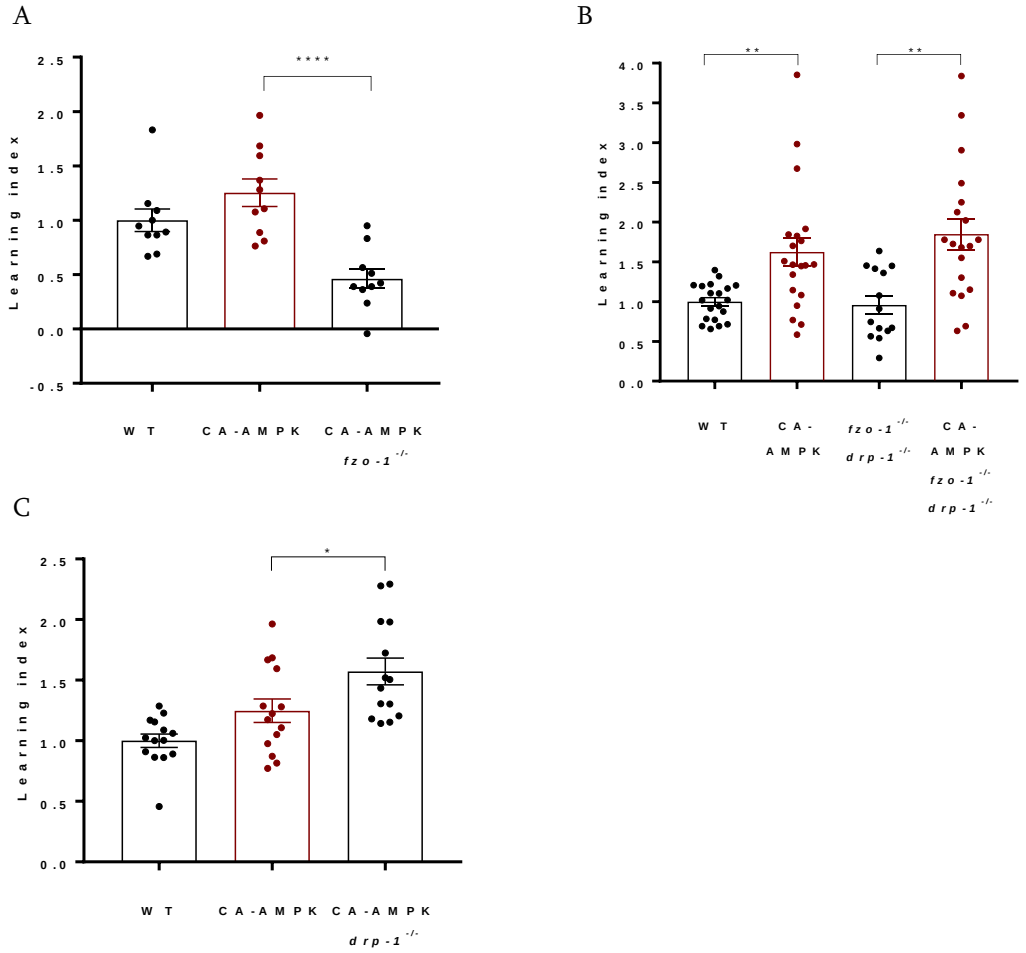

Figure 4.4: AMPK requires mitochondrial fusion to enhance neuronal plasticity

A) Memory assay performed at day1 on WT vs. CA-AMPK vs. CA-AMPK x *fzo-1*$^{-/-}$ worms. Means: (WT) 1±0.10, (CA-AMPK) 1.25±0.13, (CA-AMPK x *fzo-1*$^{-/-}$) 0.46±0.09. t-test CA-AMPK vs. CA-AMPK x *fzo-1*$^{-/-}$ $p<0.0001$. 2 independent biological replicates.

B) Memory assay performed at day1 on WT vs. *drp-1*$^{-/-}$ x *fzo-1*$^{-/-}$ vs. CA-AMPK vs. CA-AMPK x *drp-1*$^{-/-}$ x *fzo-1*$^{-/-}$ worms. Means: (WT) 1±0.05, (*drp-1*$^{-/-}$ x *fzo-1*$^{-/-}$) 0.96±0.12 (CA-AMPK) 1.63±0.18, (CA-AMPK x *drp-1*$^{-/-}$ x *fzo-1*$^{-/-}$) 1.85±0.19. t-test WT vs. CA-AMPK $p=0.0017$, t-test *drp-1*$^{-/-}$ x *fzo-1*$^{-/-}$ vs. CA-AMPK x *drp-1*$^{-/-}$ x *fzo-1*$^{-/-}$ $p=0.0011$. 2 independent biological replicates.

C) Memory assay performed at day1 on WT vs. CA-AMPK vs. CA-AMPK x *drp-1*$^{-/-}$ worms. Means: (WT) 1±0.05, (CA-AMPK) 1.25±0.10, (CA-AMPK x *drp-1*$^{-/-}$) 1.57±0.11. t-test CA-AMPK vs. CA-AMPK x *drp-1*$^{-/-}$ $p=0.0354$. 3 independent biological replicates.

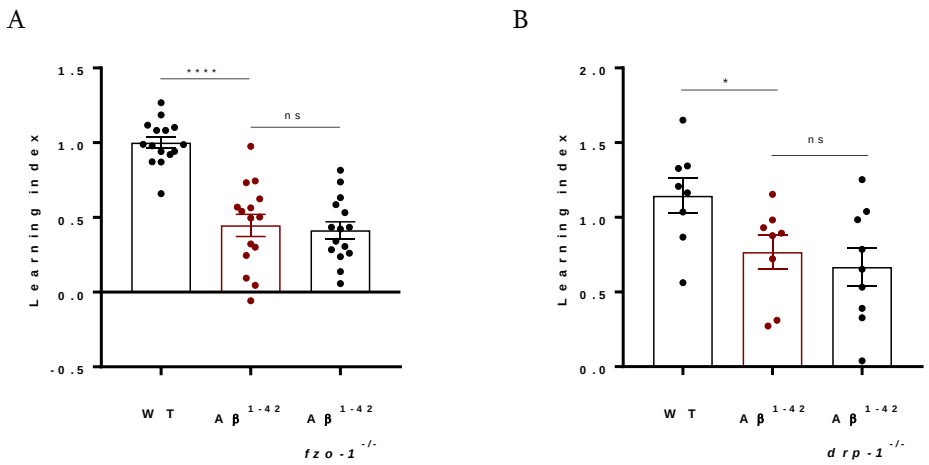

Figure 4.5: Modulation of mitochondrial morphology does not mitigate Aβ$^{1-42}$ induced deficits

A) Memory assay performed at day1 on WT vs. Aβ$^{1-42}$ vs. Aβ$^{1-42}$ x *fzo-1$^{-/-}$* worms. Means: (WT) 1±0.04, (Aβ$^{1-42}$) 0.45±0.07, (Aβ$^{1-42}$ x *fzo-1$^{-/-}$*) 0.41±0.06. t-test WT vs. Aβ$^{1-42}$ p<0.0001, t-test Aβ$^{1-42}$ vs. Aβ$^{1-42}$ x *fzo-1$^{-/-}$* p=0.7287. 3 independent biological replicates.

B) Memory assay performed at day1 on WT vs. Aβ$^{1-42}$ vs. Aβ$^{1-42}$ x *drp-1$^{-/-}$* worms. Means: (WT) 1.15±0.12, (Aβ$^{1-42}$) 0.77±0.11, (Aβ$^{1-42}$ x *drp-1$^{-/-}$*) 0.67±0.13. t-test WT vs. Aβ$^{1-42}$ p=0.0355, t-test Aβ$^{1-42}$ vs. Aβ$^{1-42}$ x *drp-1$^{-/-}$* p=0.5669. 2 independent biological replicates.

The observation that induction of mitochondrial fragmentation via *fzo-1$^{-/-}$* deletion is detrimental is not surprising given that alteration of the mitochondrial fusion process is associated with neurological disorders[258]. However, the observation that the mutant strain deleted for both *fzo-1$^{-/-}$* and *drp-1$^{-/-}$* displays a WT phenotype is surprising and novel. Mitochondria are very dynamic organelles and work by others have suggested that their dynamic capacity is key to their function, especially in the neurons and at the synapse. Here we show that the fusion/fission

process is not required in young animals to form associative learning and that the overall cellular morphology of the mitochondria is more important than its capacity to adapt through fusion or fission. Although this conclusion might be counterintuitive, it correlates with *Weir et al.*'s observation that the double mitochondrial mutant is significantly longer lived. Whether the mechanism by which the double mitochondrial mutant extends lifespan is similar to the mechanism mediating behavioral plasticity remains to be elucidated. Although AMPK seems to require a balanced mitochondrial network both for longevity and promotion of behavioral plasticity, some differences exist between the mitochondrial network states required for the longevity effect of AMPK and its role in learning; *drp-1*$^{-/-}$ blocks AMPK mediated lifespan extension but shows an additive beneficial effect on neuronal plasticity. This set of experiments cannot distinguish between a direct requirement of mitochondrial fusion in neurons downstream of AMPK versus an indirect requirement of mitochondrial fusion mediating AMPK's beneficial systemic effects. Indeed, *fzo-1* deletion blocks AMPK mediated longevity and could therefore block all beneficial effects of AMPK including those impacting behavioral plasticity as we demonstrated in our model. Although *drp-1* mutation blocks AMPK mediated longevity[135], it does not block the beneficial

effect of AMPK on behavioral plasticity, suggesting differential requirements for mitochondrial network state downstream of AMPK activation and potential tissue specificity of these requirements.

To test this hypothesis, we could examine whether AMPK can directly modulate mitochondrial morphology in neurons, and the requirement of mitochondrial fusion specifically in the nervous system for AMPK mediated enhancement of neuronal plasticity. Finally, if mitochondrial fusion is indeed mediating AMPK rescue effect on the AD strain, we hypothesized that inducing a fused mitochondrial network through *drp-1* deletion might prove beneficial. Induction of a fused mitochondrial network through *drp-1* deletion was not sufficient to rescue $A\beta^{1-42}$ induced neuronal dysfunction. Because we did not verify that *drp-1* mutation could indeed drive neuronal mitochondrial fusion in the AD strain, this result should be interpreted with caution. However, if *drp-1* mutation were effectively inducing mitochondrial fusion, it would suggest that although mitochondrial fusion is required downstream of AMPK, it is not sufficient to protect against the toxicity of the amyloid peptide. Another approach to test this hypothesis would be to

look at the effect of *drp-1*$^{-/-}$ on age related decline in behavioral plasticity to ask if it is sufficient to recapitulate the beneficial effect of CA-AMPK.

4.2. AMPK modulates neuronal mitochondrial morphology and requires fusion in the neurons for learning

To ask whether the behavioral changes we observed were due to specific modulation of neuronal biology versus a systemic effect, we moved from ubiquitous AMPK expression and whole-body deletion mutants to neuronal tissue-specific transgenes. To assess changes in neuronal mitochondrial network morphology *in vivo*, we generated a *C. elegans* strain expressing GFP targeted to the outer mitochondrial membrane specifically in neurons (**Fig4.6**).

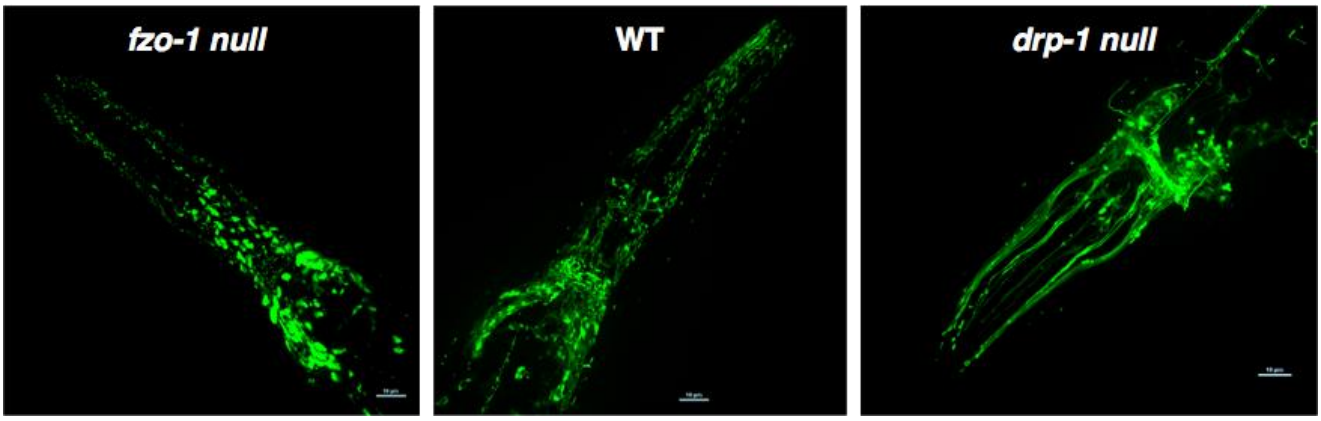

Figure 4.6: Generating a pan neuronal mitochondrial reporter in *C. elegans*

The neuronal mitochondrial reporter expresses extrachromosomal TOM20:GFP marker under the pan neuronal promoter *rab-3p.* The strain was then crossed into the mitochondrial mutants *fzo-1*$^{-/-}$ and *drp-1*$^{-/-}$. Images of OP50-1 fed worms were taken at day1 as described in *Materials&Methods*.

Several neuronal mitochondrial reporters exist in *C. elegans* however they are expressed in specific subsets of neurons[295]. Here, we wished to study overall neuronal mitochondrial morphology and therefore generated a new pan neuronal reporter, tagging with GFP the transmembrane domain of the outer mitochondrial membrane protein TOMM-20 (cf. *Materials&Methods*). In young animals (day 1 of adulthood), mitochondria appeared connected and tubular and were visible both in the soma, surrounding the nucleus as well as in the dendrites projecting to the tip of the nose (**Fig4.6** center image). To validate our reporter, we analyzed neuronal mitochondrial morphology in the mitochondrial dynamics mutants (**Fig4.6** left and right images). As expected, deletion of the outer mitochondrial membrane fusion factor *fzo-1* induced hyper-fragmentation of the network especially visible in the dendrites, as well as accumulation of aggregated signal in the soma region (**Fig4.6** left image). In contrast, deletion of the fission factor *drp-1* caused hyper-fusion of the mitochondria, which appear to form long tubules along the whole length of the dendrites (**Fig4.6** right image). These data demonstrate the validity and dynamic range of our reporter strain.

As previously reported by other groups, neuronal mitochondrial networks become significantly more fragmented with age, as illustrated by images at taken at day 8 (**Fig4.7A**). Age

induced fragmentation of the neuronal mitochondrial network correlates with the age-induced decline in behavioral plasticity in WT nematodes as well as the impaired behavioral response of *fzo-1* mutant animals. To test the tissue specificity of this effect, we rescued mitochondrial dynamics specifically in the neurons by expressing *fzo-1* under a pan-neuronal promoter (*rab-3* promoter) in a *fzo-1* deletion background (**Fig4.7B**).

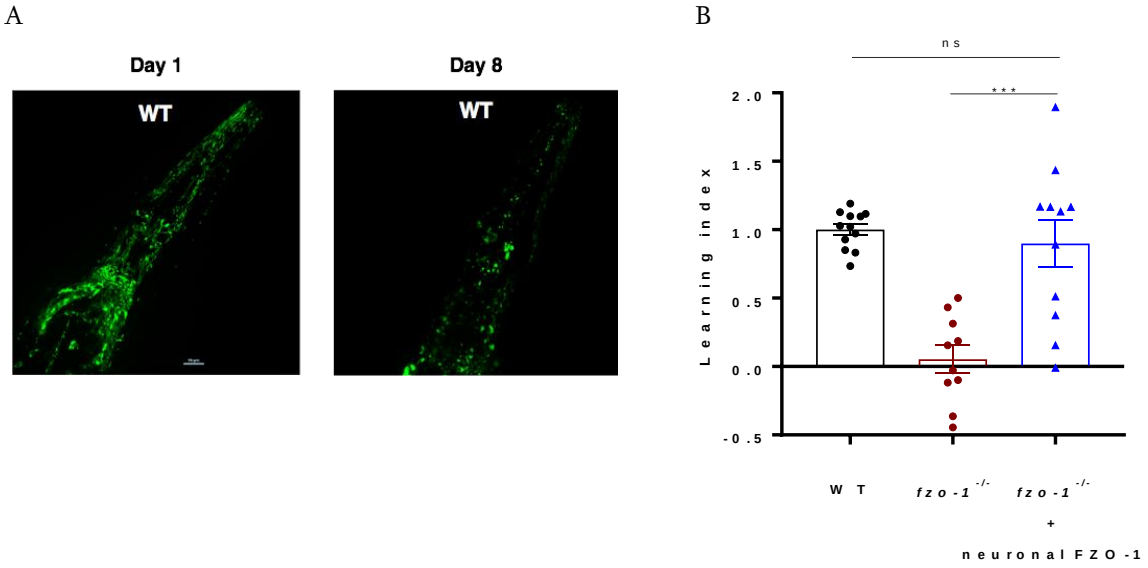

Figure 4.7: Mitochondrial fragmentation in the neurons causes the loss of behavioral plasticity

A) Representative confocal images of the neuronal mitochondrial reporter strain at day1 vs. day8 on OP50-1 bacteria. Images were taken as described in *Materials&Methods.*

B) Memory assay performed at day1 on WT worms vs. *fzo-1*$^{-/-}$ worms vs. *fzo-1*$^{-/-}$ + neuronal FZO-1 rescue worms. Means: (WT) 1±0.04, (*fzo-1*$^{-/-}$) 0.05±0.10, (*fzo-1*$^{-/-}$ + neuronal FZO-1 rescue) 0.90±0.17. t-test *fzo-1*$^{-/-}$ vs. *fzo-1*$^{-/-}$ + neuronal FZO-1 rescue p=0.0006, t-test WT vs. *fzo-1*$^{-/-}$ + neuronal FZO-1 rescue p=0.0006. 3 independent biological replicates.

Expression of neuronal FZO-1 fully rescued the learning deficit observed in fusion deficient nematodes, suggesting that mitochondrial fusion is indeed required in neurons to promote behavioral plasticity. As described previously, AMPK promotes mitochondrial fusion in muscles and intestine in *C. elegans*[294]. Given the requirement for connected mitochondrial network downstream of AMPK to enhance neuronal plasticity, we asked whether AMPK could directly induce mitochondrial fusion in neurons. Worms treated from adulthood with the pharmacological AMPK activator phenformin, not only maintained a more tubular neuronal mitochondrial network and displayed reduced age-related fragmentation of the mitochondria but also drove a more fused mitochondrial network compared to WT day 1 controls (**Fig4.8A,B**). Systemic activation of AMPK through phenformin treatment thus prevents age related changes in the mitochondrial network and further drives mitochondrial fusion, correlating with its beneficial effects on neuronal plasticity. Restoring *fzo-1* expression in neurons of CA-AMPK worms deficient in fusion capacity partially rescued the beneficial effects of AMPK activation on behavioral plasticity (**Fig4.8C**). Taken together, these results causally link AMPK beneficial effect on behavioral plasticity to modulation of neuronal mitochondrial networks in *C. elegans*.

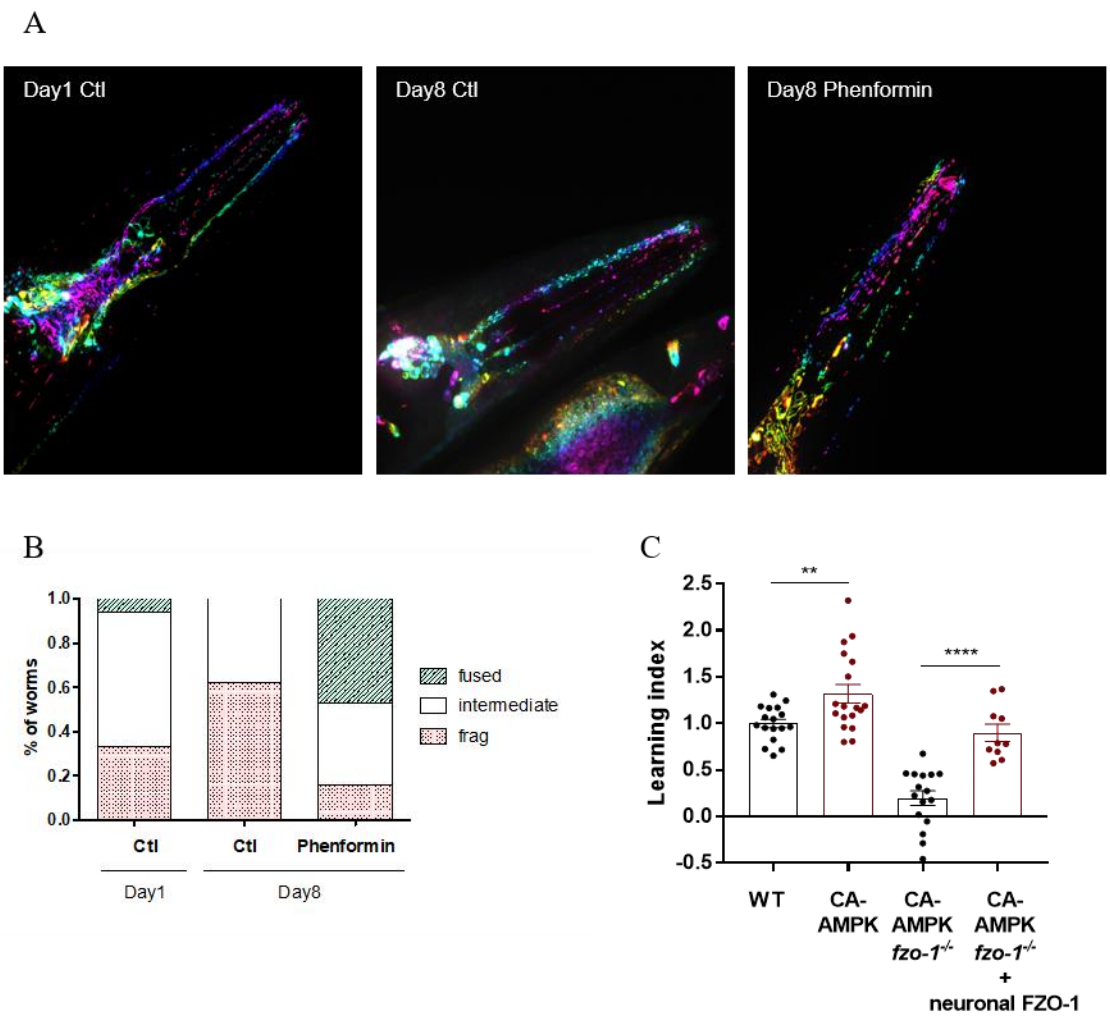

Figure 4.8: AMPK requires neuronal mitochondrial fusion to enhance neuronal plasticity

A) Representative confocal images of the neuronal mitochondrial reporter strain at day1 vs. day8 treated with Phenformin started at day1. Images were taken as described in *Materials&Methods.*

B) Quantification of the images from Fig4.5A. Images were analyzed as described in *Materials&Methods* between fused/intermediate/fragmented categories. Percentage of worms is plotted from 2 independent biological replicates.

C) Memory assay performed at day1 on WT vs. CA-AMPK vs. CA-AMPK x *fzo-1*$^{-/-}$ vs. CA-AMPK x *fzo-1*$^{-/-}$ + neuronal FZO-1 rescue worms. Means: (WT) 1±0.05, (CA-AMPK) 1.32±0.10 (CA-AMPK x *fzo-1*$^{-/-}$) 0.12±0.10, (CA-AMPK x *fzo-1*$^{-/-}$ + neuronal FZO-1 rescue) 0.90±0.09. t-test WT vs. CA-AMPK p=0.008, t-test CA-AMPK x *fzo-1*$^{-/-}$ vs. CA-AMPK x *fzo-1*$^{-/-}$ + neuronal FZO-1 rescue p<0.0001. 4 independent biological replicates.

Here we demonstrate the requirement of neuronal mitochondrial fusion for the beneficial effect of AMPK on neuronal plasticity and we previously showed that AMPK activation could fully rescue $A\beta^{1-42}$ deleterious effects. The next step is therefore to ask if modulation of neuronal mitochondrial morphology is required downstream of AMPK to mediate its beneficial effects on this neurodegenerative disease model. To do so we initially plan to study mitochondrial morphology in the AD strain to see if it displays increased fragmentation and ask if phenformin can promote mitochondrial fusion in neurons. The advantage of using a pharmacological activator of AMPK is the ability to introduce it at late onset. In the experiments described here, phenformin treatment enhanced neuronal plasticity when administered from hatch, thus mimicking the genetic activation if AMPK. We showed, by analyzing neuronal mitochondrial morphology of worms with adult onset phenformin treatment, that phenformin given later in life reserve age related phenotypes. As a future experiment, we could first study the effect of late onset phenformin treatment on WT worms cognitive decline and ask whether initiating phenformin treatment in AD strain at a time point where worms already display an impaired behavioral response can reverse the deficit. This experiment is actually more relevant to the human disease where a treatment is

usually initiated after the disease diagnosis. To further test this hypothesis, we should determine whether the low $A\beta^{1-42}$ level expression of our AD strain is sufficient to provoke neuronal cell death. Indeed, if $A\beta^{1-42}$ induces early on neuronal death which potentially explains the cognitive deficits, late onset phenformin might prevent further decline in neuronal dysfunction rather than reverse the phenotype. In contrast, if our AD strain does not display early on neuronal cell death, then late onset phenformin might be able to completely reverse the neuronal plasticity impairment by modulating cellular metabolism, possibly through mitochondrial morphology. All of these hypotheses remain to be tested.

To ask whether activation of AMPK had a beneficial effect on *C. elegans* proteotoxic stress models in general or a more specific action on $A\beta^{1-42}$ induced neuronal toxicity, we turned towards another degeneration model in *C. elegans*, a Huntington's Disease model. These strains express specifically in the neurons, peptides carrying different number of repeats of glutamine (Q): the control strain Q0 and the disease model strains Q40 and Q67[225]. Previous labs have demonstrated

that a number of 40 CAG repeats is the threshold at which the protein shifts from being soluble in the cytoplasm to insoluble and aggregated[225] (**Fig4.9A**).

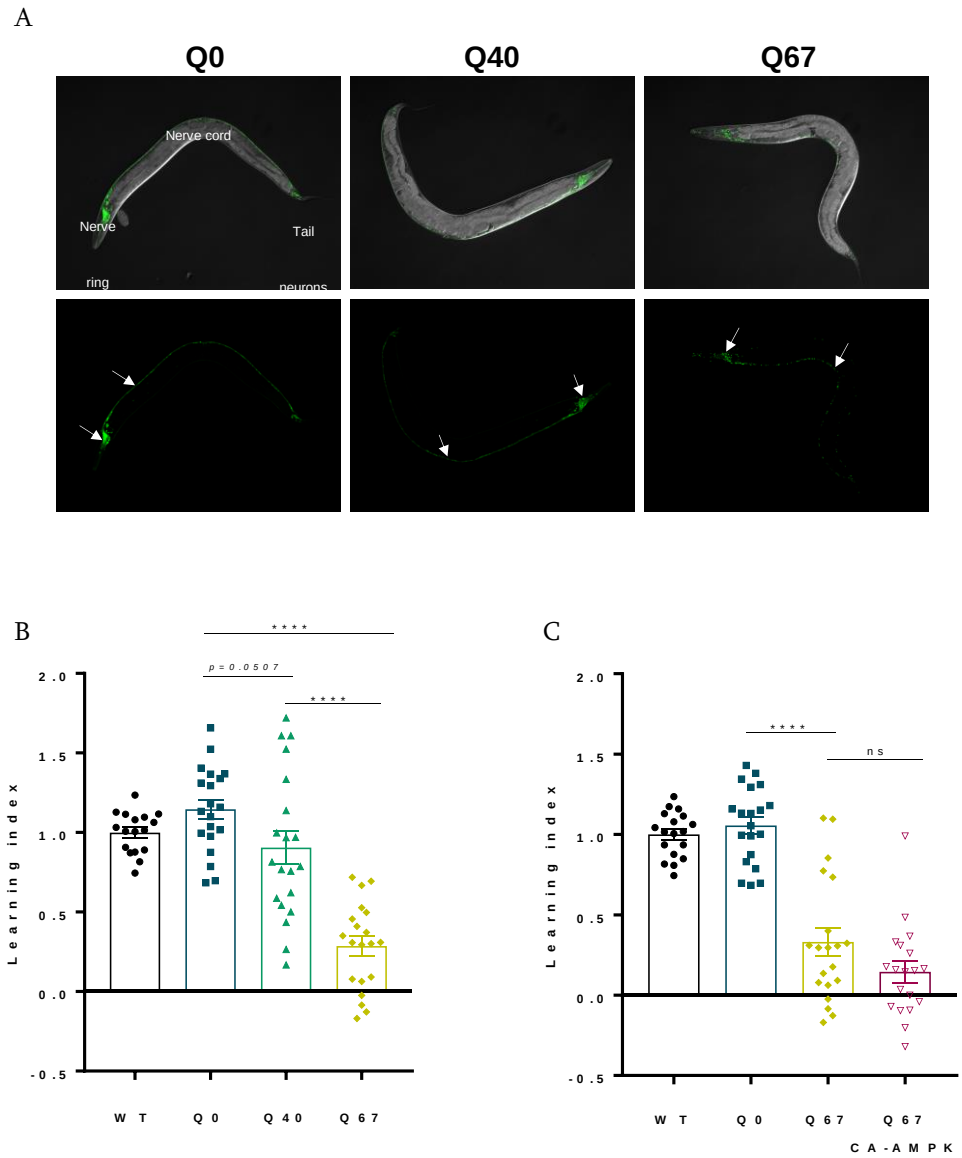

Figure 4.9: CA-AMPK does not rescue mutant Htt induced behavioral defects

A) Representative images of polyQ strains showing CAG repeats-dependent aggregation in day1 worms fed on OP50-1 *E. Coli*. Arrows point to either diffuse GFP (left image) or aggregated GFP signal (right image) in the nerve ring or along the nerve cord.

B) Memory assay performed at day1 on WT vs. polyQ0 vs. polyQ40 vs. polyQ67 worms. Means: (WT) 1±0.03, (polyQ0) 1.15±0.06 (polyQ40) 0.91±0.10, (polyQ67) 0.29±0.06. t-test polyQ0 vs. polyQ40 p=0.0507, t-test polyQ0 vs. polyQ67 p<0.0001, t-test polyQ40 vs. polyQ67 p<0.0001. 4 independent biological replicates.

C) Memory assay performed at day1 on WT vs. polyQ0 vs. polyQ67 vs. polyQ67 x CA-AMPK worms. Means: (WT) 1±0.03, (polyQ0) 1.06±0.05 (polyQ67) 0.33±0.08, (polyQ67 x CA-AMPK) 0.15±0.07. t-test polyQ0 vs. polyQ67 p<0.0001, t-test polyQ67 vs. polyQ67 x CA-AMPK p=0.0988. 4 independent biological replicates.

Rather than looking at paralysis as a readout of neuronal dysfunction as has been done with this model, we decided to test whether aggregation of polyQ proteins could induce a behavioral plasticity deficit. As shown in **Fig4.9B**, the number of polyglutamine repeats correlates with neuronal plasticity impairment suggesting a correlation between the extent of the aggregation and the neuronal dysfunction. The polyQ40 strain, which displays mild to no aggregation of the GFP labeled polyQ peptide (**Fig4.9A**) shows a small but not significant behavioral plasticity deficit. The polyQ67 shows a very significant loss of neuronal plasticity, similar to the results obtained with the AD model (**Fig4.9B**). Genetic activation of AMPK (CA-AMPK), however, did not rescue this behavioral response (**Fig4.9C**), suggesting that AMPK activation does not broadly reverse proteotoxic stress but rather has a beneficial effect specific to $A\beta^{1-42}$ induced neuronal toxicity.

Indeed, despite the fact that both our AD and HD model strains represent proteotoxic stress models with neuronal expression of aggregated proteins, the same intervention, AMPK activation, does not equally benefit both disease models. This result would suggest that different cellular functions are altered downstream of $A\beta^{1-42}$ or polyQ67 accumulation and that AMPK mediates its effect on those downstream processes. AMPK activation does not represent a universal intervention to treat various toxic aggregation disorders but seems to be more specific to the physiopathology of AD. These observations nicely correlate which human and mice striatum data where AMPK was found to be abnormally activated and further contributing to oxidative stress and HTT induced neuronal cell death[163].

5. Chapter V Targeting CRTC-1 (CREB regulated transcription coactivator 1), a downstream mediator of AMPK

Recent work in *C. elegans* has defined the CRTCs as novel modulators of aging that link energy sensing to transcriptional regulation of longevity[79,296]. Expression of a constitutively active AMPK increases lifespan in *C. elegans*, while also promoting phosphorylation and inhibition of CRTC-1. Expression of a constitutively active CRTC-1 specifically in the nervous system of *C. elegans*, by mutation of the AMPK phosphorylation sites (Serine) to Alanine is sufficient to block AMPK mediated lifespan extension[296] (**Fig5.1**). Supporting a role for CRTCs inhibition in aging, inactivation of CRTC-1 via RNAi is sufficient to extend lifespan in a CREB-dependent manner[79].

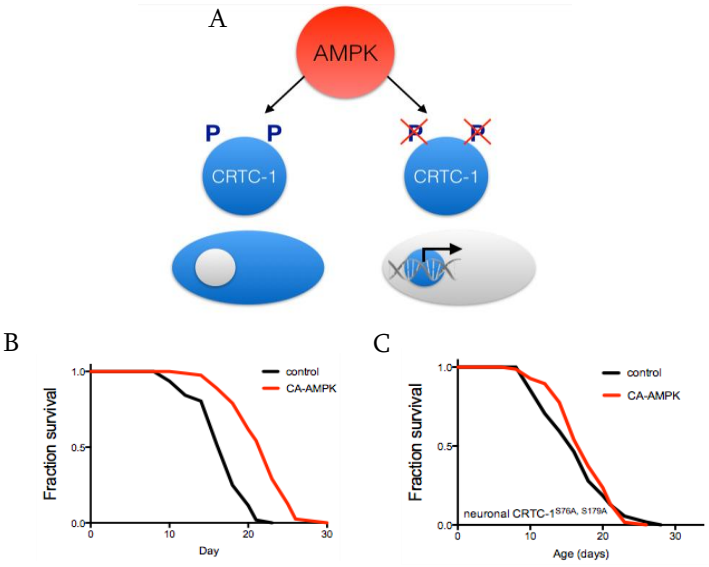

Figure 5.1: AMPK inhibits CRTC-1 in neurons to extend lifespan. ***Adapted from Burkewitz et al.***[296]

A) Schematic of AMPK neuronal regulation of neuronal CRTC-1 in *C. elegans*

B) Representative lifespan of WT vs. CA-AMPK animals showing lifespan extension by ubiquitous overexpression of CA-AMPK. From *Burkewitz et al.*
C) Representative lifespan of WT vs. CA-AMPK animals expressing neuronal CRTC-1 S>A mutations showing blocked lifespan extension by CA-AMPK. From *Burkewitz et al.*

5.1. Introduction

Initially named transducers of regulated CREB (TORCs) or mucoepidermoid carcinoma translocated protein (MECTs), CRTC family members were first identified as coactivators of the transcription factor CREB. However, recent work has identified expanding roles for CRTCs outside the confines of their name, showing that CRTCs act both as regulators of transcription factors beyond CREB and of processes other than transcription[1]. Three isoforms of CRTC (CRTC1–3) have been described in mammals, with CRTC2 and 3 showing 32% homology with CRTC1[297]. In humans, CRTC2 and CRTC3 are ubiquitously expressed, whereas CRTC1 is mainly expressed in the brain, with little expression in other tissues[298-301]. One homolog, *crtc-1*, exists in *C. elegans* and its expression has been characterized in the nervous system and the intestine[79,296]. The connections between the CRTCs and age-related pathologies are numerous and deregulation of various isoforms of CRTC has been causally liked to major disorders such as cancer, metabolic

disease and neurodegeneration. In my PhD work, I focused on CRTC as a downstream effector of AMPK and studied its role in the context of brain aging and neurodegeneration.

CREB has an evolutionarily conserved fundamental role in regulating memory formation and memory consolidation[302-304]. The cAMP–CREB pathway controls memory for example by enhancing synaptic transmission or increasing neuronal excitability[302]. Modulating CREB levels or its binding partner the histone acetyltransferase CBP (CREB binding protein) by overexpression or knockout results in either enhanced memory formation or memory deficits, respectively, in mammalian models[302]. Even though CREB phosphorylation at Ser133 is an important activating post-translational modification, it is not always sufficient to induce CREB dependent gene transcription[305], suggesting that additional mechanisms regulate CREB function. CRTC1 has therefore been mainly studied in the context of memory formation and has been characterized for its role in neurodegenerative diseases[306].

Intriguingly, in brain tissue, activation of CRTC1 seems to be beneficial, in contrast to the detrimental effects of CRTC hyperactivation observed in metabolic disease and cancer[1]. Crtc1 knockout mice do not display any brain developmental defects[299]; however, these mouse models have been informative in highlighting the importance of CRTC1 in neuronal function[307,308]. Crtc1 knockout mice display a treatment-resistant depression-like phenotype[308] as well as symptoms associated with mood disorders such as impulsive aggressiveness, social withdrawal, and decreased sexual motivation. These phenotypes are likely caused by decreased levels of dopaminergic and serotoninergic activity in the mouse prefrontal cortex, and by reduced expression of CREB susceptibility genes involved in neuroplasticity[307]. Synaptic activity plays a major role in regulating CRTC1 function in neurons. Synaptic stimuli induced by AMPA (a-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate) and NMDA (Nmethyl-D-aspartic acid)-type glutamate receptors, LVGCC (L-type voltage-gated calcium channels) calcium influx, or GABA (g-aminobutyric acid) receptor activation are necessary to trigger CRTC1 nuclear import and transcription-dependent plasticity effects. Indeed, CRTC1 localizes to dendrites and spines in electrically silenced hippocampal neurons and translocates to the nucleus in a calcium- and

calcineurin-dependent manner upon stimulation[309]. Posttranslational modifications and subsequent changes in CRTC1 subcellular localization integrate this signal. Activation of synaptic NMDA or AMPA glutamate receptors, or of LVGCC, induces a local increase in calcium concentration at the synapse (as opposed to a general rise in intracellular calcium), which triggers the dynein-mediated retrograde transport of CRTC1 along microtubules from the synapse to the nucleus[310]. The activity-dependent nuclear shuttling of CRTC1 in neurons occurs within minutes and, conversely, once the stimulus stops, CRTC1 returns to the cytoplasm in 30 minutes[311]. In hippocampal neurons, basal constitutive phosphorylation of CRTC1 at Ser151 and Ser245 is controlled by SIK2/AMPK and MARK2 enzymes, whereas stimulus-triggered dephosphorylation is mediated by the phosphatase calcineurin[311]. Dephosphorylation of three serine residues (Ser64, Ser151, Ser145) on CRTC1 is necessary and sufficient for dissociation from 14-3-3 protein and nuclear accumulation[310]. Activity-stimulated dephosphorylation of CRTC1 at Ser151 can induce transcriptional activation of CRTC1 in mouse hippocampus[312]. Nuclear CRTC1 then drives CRE-dependent transcription even in the absence of CREB phosphorylation on Ser133[311]. However, CREB is required for CRTC1 to bind to CRE sites in the promoters of immediate early genes

(IEG) in an activity-dependent manner. Long-term potentiation (LTP) is an activity-dependent long-lasting increase in synaptic strength. LTP is a form of synaptic plasticity which requires gene transcription and protein synthesis and is considered to be an electrophysiological model for the basic mechanisms involved in learning and memory formation[313,314]. CRTC1 in neurons senses the coincidence of calcium and cAMP signals and induces a CREB-dependent transcriptional response leading to enhanced synaptic transmission (through, for example, the expression of brain-derived neurotrophic factor, BDNF)[300,315,316]. CRTC1 is involved in the maintenance of late-phase LTP in mouse hippocampal slices[300], and induction of late-phase LTP triggers robust nuclear accumulation of CRTC1 in neurons. Consistently, nuclear localization of CRTC1 facilitates late-phase LTP induction in mouse hippocampal slices, whereas expression of a dominant negative CRTC1 suppresses the maintenance of late-phase LTP[315,317]. Similarly, increasing CRTC1 or CREB function in excitatory dentate granule cells of the dentate gyrus region of the dorsal hippocampus of mice is sufficient to enhance memory stabilization as well as memory reactivation without compromising memory quality in a fear conditioning assay, suggesting a region-specific role for CRTC1 in controlling memory[318]. More recent work has shown that, in mouse

hippocampus, CRTC1 induces chromatin changes following associative learning to promote sustained target gene transcription and enhance memory strength[319]. In Drosophila, Crtc nuclear activity is required specifically in the mushroom body of the fly for memory formation induced in appetitive long-term memory (fLTM: a single training preceded by a period of fasting). The fasting period downregulates the insulin signaling pathway in Drosophila, which normally phosphorylates Crtc via SIK2 leading to Crtc degradation[320]. Nuclear translocation of Crtc after fasting can be mimicked by the expression of a constitutively active form of Crtc, which is necessary and sufficient to form fLTM in the mushroom body. Supporting this mechanism, Drosophila chico mutants that display reduced insulin signaling mimic fLTM through the activation of Crtc in the mushroom body[321]. Interestingly, the Drosophila chico mutation, which extends lifespan via downregulation of the insulin signaling pathway[322], has been shown to activate Crtc to promote beneficial effects[320,323]. These observations contrast with the requirement for CRTC-1 inhibition in AMPK-mediated longevity in *C. elegans*[79], suggesting that CRTCs might display opposing roles in the regulation of lifespan or metabolism, and in neuronal function and memory. Lastly, in addition to being involved in the formation of memory in Drosophila, the CRTC/CREB complex

is also involved in early maintenance of long-term memory (LTM), targeting genes required for memory maintenance and extinction in mushroom bodies[321]. Data in mammals and Drosophila therefore highlight the importance of CRTC in neuronal plasticity and memory formation.

Not surprisingly, therefore, CRTC deregulation has been linked to the pathogenesis of age-onset diseases associated with memory defects such as AD and Huntington's disease. In the $APP^{sw/ind}$ mouse model of AD (transgenic mice expressing the mutant human APP695 isoform harboring the familial AD-linked Swedish K670N/M671L and Indiana V717F mutations in neurons), accumulation of the toxic amyloid β peptide in neurons reduces calcineurin activity by disrupting calcium influx through L-type calcium channels. Calcineurin-dependent CRTC1 dephosphorylation and activation is thus impaired, leading to disruption of the CRTC1-dependent gene expression program. On a functional level, the $APP^{sw/ind}$ mice display a hippocampus-dependent memory deficit in the Morris water maze spatial memory task, and this correlates with a specific reduction in the expression of CRTC1-dependent genes normally upregulated during training[324]. Furthermore, overexpression of CRTC1 by AAV (adeno-associated virus) delivery in

mouse hippocampus improves learning and memory of the AD mice in the Morris water maze test, without affecting the performance of wild-type animals[312]. Human data support the hypothesis that CRTC1 function is altered in AD patients and might therefore represent a therapeutic target. Studies reveal a reduction of both total and phosphorylated CRTC1, as well as decreased expression of CRTC1 targets, in human hippocampal samples of AD pathological cases (classified according to Braak stages)[312,325]. In addition, DNA methylation at the CRTC1 promoter is significantly lower in hippocampus samples of AD patients, and methylation levels inversely correlate with phospho-tau and β-amyloid pathology, suggesting a link between CRTC1 activity and neurodegenerative pathophysiology.

Huntington's Disease (HD) is caused by an abnormal autosomal dominant glutamine repeat expansion of 3 nucleotides (CAG) in the huntingtin (HTT) protein, leading to cognitive, psychiatric, motor dysfunction, and loss of autonomy during the final stages of the disease (Huntington's Disease Society of America; www.hdsa.org). Similar to Alzheimer's Disease, CRTC1 expression levels are decreased *in vivo* in postmortem striatal tissue from HD patients, in

the striatum of various transgenic mice models (NLS-N171-82Q, R6/2 and HdhQ111 HD), and *in vitro* in STHdhQ111 cells expressing mutant HTT[326]. Mutant HTT interacts with SIRT1 and disrupts SIRT1- mediated activation of CRTC1[327]. Under normal conditions, SIRT1 promotes deacetylation of CRTC1 (Lysines 13 and 20) and activates CRTC1 by promoting its dephosphorylation and interaction with CREB, which leads to activation of neuronal target genes such as Bdnf. Interestingly, this activating effect of SIRT1 on CRTC1 in neurons contrasts with the effect of SIRT1 on CRTC2 in the liver. Indeed, fasting induces SIRT1-mediated deacetylation of CRTC2 and its subsequent ubiquitination and degradation by the proteasome[76,328]. Such opposing regulation by SIRT1 of different CRTC isoforms might indicate tissue-specific modulation and physiological roles for the CRTCs. In a mouse model of HD (R6/2), SIRT1 overexpression is neuroprotective, and CRTC1 and CREB are at least partially required for SIRT1-mediated neuroprotection in primary cortical neurons. The requirement for CRTC1 in SIRT1-mediated neuroprotection *in vivo* in a HD mouse model remains to be explored[327]. CRTC plays an important role in the expression of an additional key metabolic regulator, the transcriptional coregulator PGC-1α (peroxisome proliferator-activated receptor g coactivator 1α)[300]. PGC-1α is a

master regulator of mitochondrial biogenesis, energy homeostasis, adaptive thermogenesis, and glucose metabolism whose expression is impaired in HD[326,329]. *Chaturvedi et al*. therefore asked whether PGC-1α might mediate the beneficial effects of CRTC1 in HD models. CRTC1 overexpression in wild-type or HTT expressing striatal cells (Htt cells) increased PGC-1α promoter activity, the expression of downstream targets (NRF-1, Tfam, CytC), as well as mitochondrial DNA content, mitochondrial activity, and mitochondrial membrane potential[326]. Consistent with this result, CRTC1 knockdown enhances mitochondrial dysfunction caused by a mitochondrial toxin (3-NP, inhibits complex II of the electron transport chain) in Htt cells, and this effect can be partially rescued by PGC-1α overexpression. Finally, specific knockdown of CRTC1 in the striatum in NLS-N171-82Q HD transgenic mice induces neurodegeneration and cell death[326].

Given the results of these studies, CRTCs represent a potential therapeutic target for various age-related diseases ranging from metabolic dysfunction to neurological disorders. However, recent work highlights the complex and sometimes opposing roles of CRTCs in aging versus

specific diseases. It is therefore necessary to better elucidate the mechanism of CRTCs activity, and how their dysregulation affects physiology and pathogenesis in aging individuals.

5.2. Results

Although the literature in mammals and human studies characterize CRTC as a protective factor in the brain tissue, it seems to contradict what one would hypothesize from the *C. elegans* data. In *C. elegans*, AMPK phosphorylates and inactivates CRTC systemically to promote healthy aging. We also demonstrated in this doctoral work the protective effects of AMPK on age and Aβ induced cognitive decline in *C. elegans*. We therefore asked if AMPK mediated its beneficial effect on behavioral plasticity through modulation of CRTC

We initially investigated the role of CRTC-1 in neurons in an AD model in *C. elegans* and chose to measure neuronal death as a readout of $A\beta^{1-42}$ induced toxicity. We used a reporter strain expressing GFP under a glutamatergic specific reporter (*eat-4* promoter), a subset of neurons vulnerable during AD progression. More specifically, we imaged the tail region of the nematodes

in which 5 distinct neurons can be visualized [cite]. We first confirmed that between day 1 and day5 of adulthood, WT animals do not display an age dependent loss of neurons. When $A\beta^{1-42}$ was constitutively co-expressed in the same group of neurons, we observed a neuronal loss already visible at day 1 of adulthood, which progressively worsened with age (**Fig5.2A**). We then examined the effect of *crtc-1* mutation on $A\beta^{1-42}$ induced neuronal death by crossing these reporter strains into a mutant strain carrying a *crtc-1* allele with a partial deletion. The frame shift occurs in the splicing domain of the protein thus disrupting the splicing domain as well as a transactivation domain of CRTC-1, rendering the protein inactive. This allele still carries the CREB binding domain and the nuclear localization sequence, however, evidence suggests that CRTCs protect against $A\beta^{1-42}$ induced toxicity through its transcriptional role. We therefore assimilated this strain as a loss of function mutant in our experiments. *Crtc-1* deletion did not impair neuronal survival in a WT background (**Fig5.2A**), however, it showed additive detrimental effects with $A\beta^{1-42}$ induced neuronal cell death (**Fig5.2A**). We then asked whether overexpression of CRTC-1, specifically in the glutamatergic neurons could alleviate $A\beta^{1-42}$ induced toxicity.

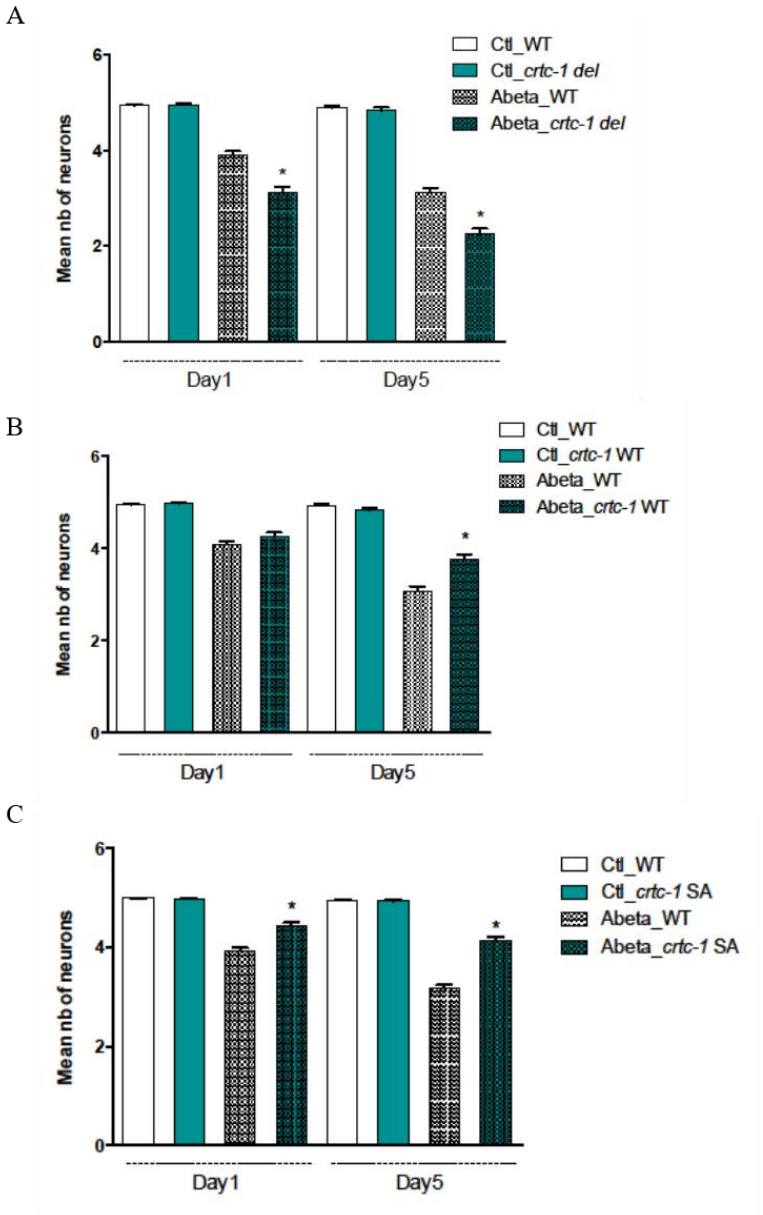

Figure 5.2: CRTC-1 protects against Aβ$^{1-42}$ induced cell death in glutamatergic neurons

A) Quantification of GFP labelled tail glutamatergic neurons in a WT and Aβ$^{1-42}$ backgrounds, +/- whole body *crtc-1* partialΔ at day 1 versus day 5. T-test (Abeta WT vs. Abeta *crtc-1* del at day1) $p<0.05$, (Abeta WT vs. Abeta *crtc-1* del at day5) $p<0.05$

B) Quantification of GFP labelled tail glutamatergic neurons in a WT and Aβ$^{1-42}$ backgrounds, +/- *eat-4p:crtc-1* (WT sequence) at day 1 versus day 5. T-test (Abeta WT vs. Abeta *crtc-1* WT at day1) p=ns, (Abeta WT vs. Abeta *crtc-1* WT at day5) $p<0.05$

C) Quantification of GFP labelled tail glutamatergic neurons in a WT and Aβ^{1-42} backgrounds, +/- *eat-4p:crtc-1* S>A at day 1 versus day 5. T-test (Abeta WT vs. Abeta *crtc-1* S>A at day1) $p<0.05$, (Abeta WT vs. Abeta *crtc-1* S>A at day5) $p<0.05$

Although we did not see a difference at day 1 of adulthood (**Fig5.2B**), CRTC-1 overexpression delayed age-related loss of glutamatergic neurons in the AD strain. Here, the WT sequence of CRTC-1 was cloned; despite the overexpression and the resultant increase in CRTC-1 activity, the transcription factor can still be subject to regulation and nuclear exclusion. We decided to clone the constitutively nuclear form of CRTC-1 [cite] which cannot be phosphorylated on its AMPK phosphorylation sites. Expression of CRTC-1 S>A in the glutamatergic neurons provided a protective effect on neuronal cell death both at day 1 and day 5 (**Fig5.2C**).

These data support the hypothesis that CRTC-1 has a protective role in neurons, especially in the context of AD. The observation that the constitutively nuclear form of CRTC-1 protects more potently against Aβ^{1-42} induced neuronal death suggests that CRTC-1 mediates its beneficial effect through its transcriptional role. Although this idea is strongly supported by additional evidence from the literature, we cannot completely exclude a non-transcriptional role for CRTC-

1 [cite review]. In the recent years, more CRTC domains have been identified, including a putative splicing domain, a cellular process which also happens inside the nucleus. Although no role for CRTC-1 in the splicing process has yet been characterized, it would be interesting to test whether a non-transcriptional function of CRTC-1 contributes to its effect on neuronal death. One caveat which should be mentioned when using this AD model is the relative sickness of the animals. Indeed, even though the $A\beta^{1-42}$ peptide is only expressed in a limited subset of neurons, these animals have a significantly shortened lifespan suggesting a more systemic toxic effect of the peptide. The important overexpression of the amyloid peptide also leads to neuronal loss visible at day 1. This could suggest a developmental issue rather than induction of cellular death after maturation of the nervous system. This problem is actually quite common in the neurodegeneration field as most of the AD mice models display very early onset neurodegenerative features. Although these models provide great insight into disease pathogenesis, they lack the age-related component of the disease progression and their use might therefore identify factors which could provide protective effect but could be irrelevant in an aged physiology setting. Finally, using this model,

we only studied a specific subset of neurons, we thus turned towards our learning and memory protocol in *C. elegans* to examine the role of CRTC-1 more broadly on cognitive functions.

In order to examine the role of CRTC-1 in behavioral plasticity in *C. elegans*, we used 2 mutant alleles: the deletion strain mutated for its transactivation domain (partialΔ) and a CRISPR generated complete knock out allele (*crtc-1* KO) with a frame shift mutation located in the beginning of the CREB binding domain (**Fig5.3A,B**). Both *crtc-1* mutants displayed an improved behavioral response in the NaCl protocol. This result is in complete contradiction with the mammalian data which suggest a beneficial role of CRTC1 in memory formation[1]. We decided to further investigate this observation by looking at the effect of activated neuronal CRTC-1 in *C elegans*. Overexpression of the constitutively activated form of *crtc-1* under a pan neuronal promoter (*rab-3*p) led to a complete impairment of the behavioral response of the animals (**Fig5.3C**) suggesting a detrimental impact of CRTC-1 activity on memory formation. This again is opposite to what the mammalian literature suggests, however, it is consistent with our previous observations in *C. elegans* using the *crtc-1* loss of function models. Despite the fact that the adult

C. elegans only has 302 neurons, its nervous system is complex and specific subgroups of neurons perform specific tasks; as an example, the pair of NaCl sensing neurons ASE have 2 distinct opposite roles as one neuron signals attraction whereas the other signals repulsion from the same stimulus. For this reason, we hypothesized that CRTC-1 activity might not be required in all neurons and overexpressing it in the entire nervous system might prove detrimental. We therefore decided to express *crtc-1* S>A in sensory neurons using the *osm-6* promoter (**Fig5.3D**). Although the difference was less pronounced, worms expressing the activated form of CRTC-1 in sensory neurons still displayed an impaired phenotype in our behavioral assay.

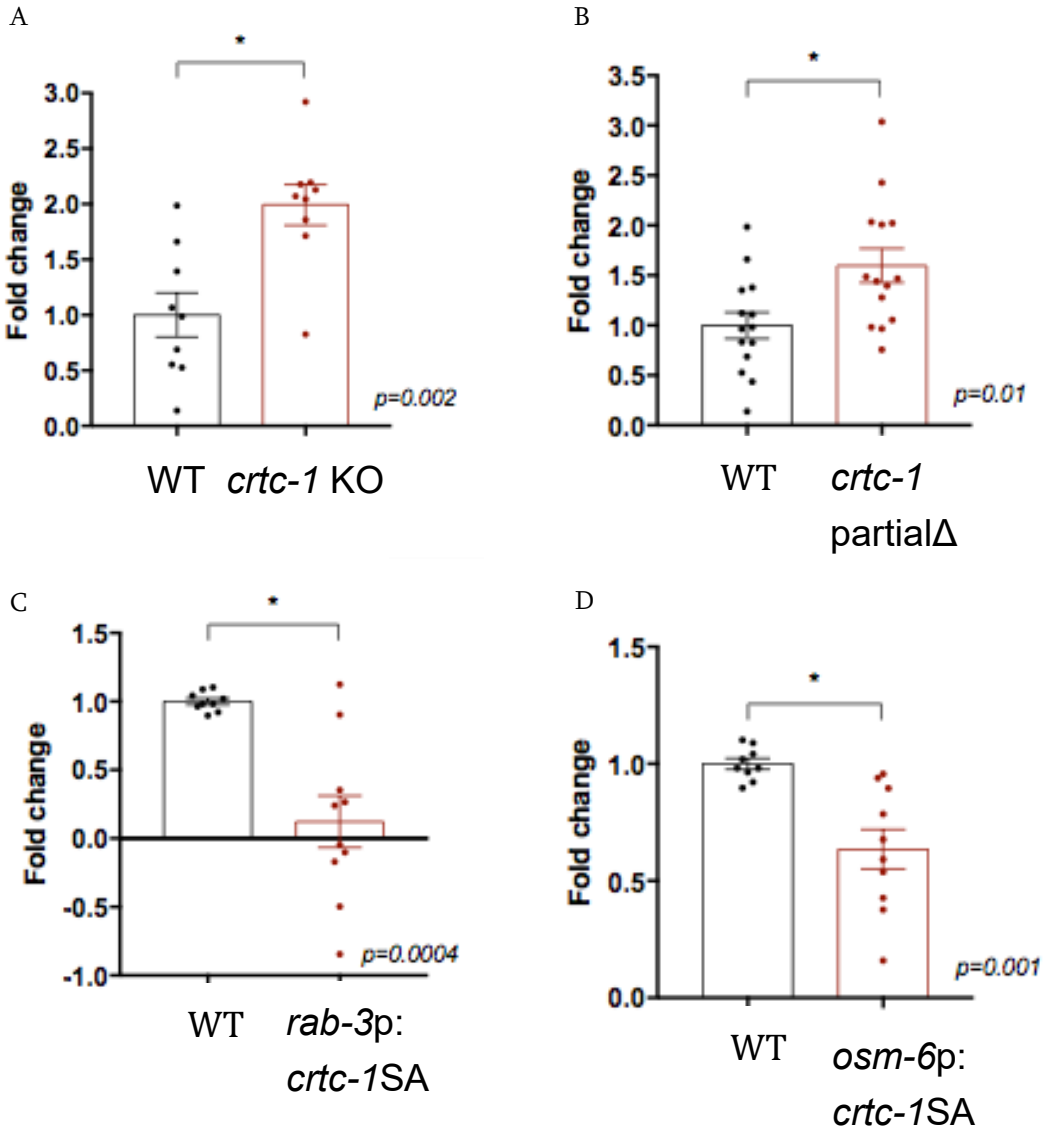

Figure 5.3: CRTC-1 activation in neurons impairs behavioral plasticity

A) Memory assay performed at day1 on WT vs. *crtc-1*$^{-/-}$ (CRISPR knock out strain) worms. Means: (WT) 1±0.19, (*crtc-1* KO) 1.99±0.18. t-test WT vs. *crtc-1* KO *p*=0.002. 2 independent biological replicates.

B) Memory assay performed at day1 on WT vs. *crtc-1* partialΔ (*crtc-1*^{tm2869}) worms. Means: (WT) 1±0.13, (*crtc-1* partialΔ) 1.59±0.17. t-test WT vs. *crtc-1* partialΔ *p*=0.01. 3 independent biological replicates.

C) Memory assay performed at day1 on WT vs. *rab-3p:crtc-1* S>A worms. Means: (WT) 1±0.02, (*rab-3p:crtc-1* SA) 0.12±0.18. t-test WT vs. *rab-3p:crtc-1* S>A *p*=0.0004. 2 independent biological replicates.

D) Memory assay performed at day1 on WT vs. *osm-6p:crtc-1* S>A worms. Means: (WT) 1±0.02, (*osm-6p:crtc-1* SA) 0.63±0.08. t-test WT vs. *osm-6p:crtc-1* S>A *p*=0.001. 2 independent biological replicates.

We have previously shown that neuronal CRTC-1 S>A blocked AMPK mediated lifespan extension and we further demonstrated the additional beneficial effect of AMPK activation on behavioral plasticity in *C. elegans*. If we hypothesize that *crtc-1* also mediates AMPK beneficial effect on neuronal functions, the data in **Fig5.3** is consistent. Indeed, we showed a beneficial effect of *crtc-1* loss of function as well as a detrimental impact of overexpression of a constitutively active form of CRTC-1. Unfortunately, these findings contradict both the effects of CRTC-1 on neuronal cell death (**Fig5.2**) as well as published literature[1]. From a technical side, further work is needed to support these findings. *Mair et al.* demonstrated the nuclear localization of CRTC-1 S>A in intestinal cells, but we have not yet confirmed here the nuclear localization of CRTC-1 in neurons by imaging. Furthermore, we expressed *crtc-1* S>A in sensory neurons to study behavioral plasticity but have not yet tested whether it affects sensing of the stimulus NaCl itself. Finally, it

would be interesting to dissect which neuronal circuits are directly relevant for CRTC-1 effect on neuronal plasticity. Understanding the exact neurons in which CRTC-1 mediates its effects would allow us to determine whether the same downstream mechanisms are involved between mediation of behavioral plasticity and protection against neuronal loss. Indeed, if CRTC-1 acts in different sets of neurons, it could explain the discrepancy we observed between its beneficial effect on neuronal death versus detrimental impact on behavioral response.

Given the directionality of the effects of CRTC-1 on behavioral plasticity in *C. elegans*, we asked if AMPK modulates CRTC-1 to promote its beneficial effects on associative learning. To test this hypothesis, we first asked if loss of function of *crtc-1* had additive effect with AMPK activation on behavioral plasticity and then tested if blocking AMPK regulation of CRTC-1 through mutation of the phosphorylation sites blocked CA-AMPK induced improved behavioral response. We did not measure a significant difference between the *crtc-1* loss of function mutants, CA-AMPK strain and the combination of both (**Fig5.4A,B**). All strains showed improved behavioral response compared to WT animals but we did not observe an additive effect of *crtc-1*

mutation and AMPK activation. We tested both *crtc-1* null alleles and obtained the same result (**Fig5.4A,B**), suggesting that AMPK and CRTC-1 might act in the same pathway to control behavioral plasticity. Expression of neuronal CRTC-1 S>A, the constitutively active form of CRTC-1, blocked AMPK mediated enhanced behavioral response (**Fig5.4C**). This result suggests that inhibition of CRTC-1 is required for the effect of AMPK on associative learning and more specifically, required in the nervous system.

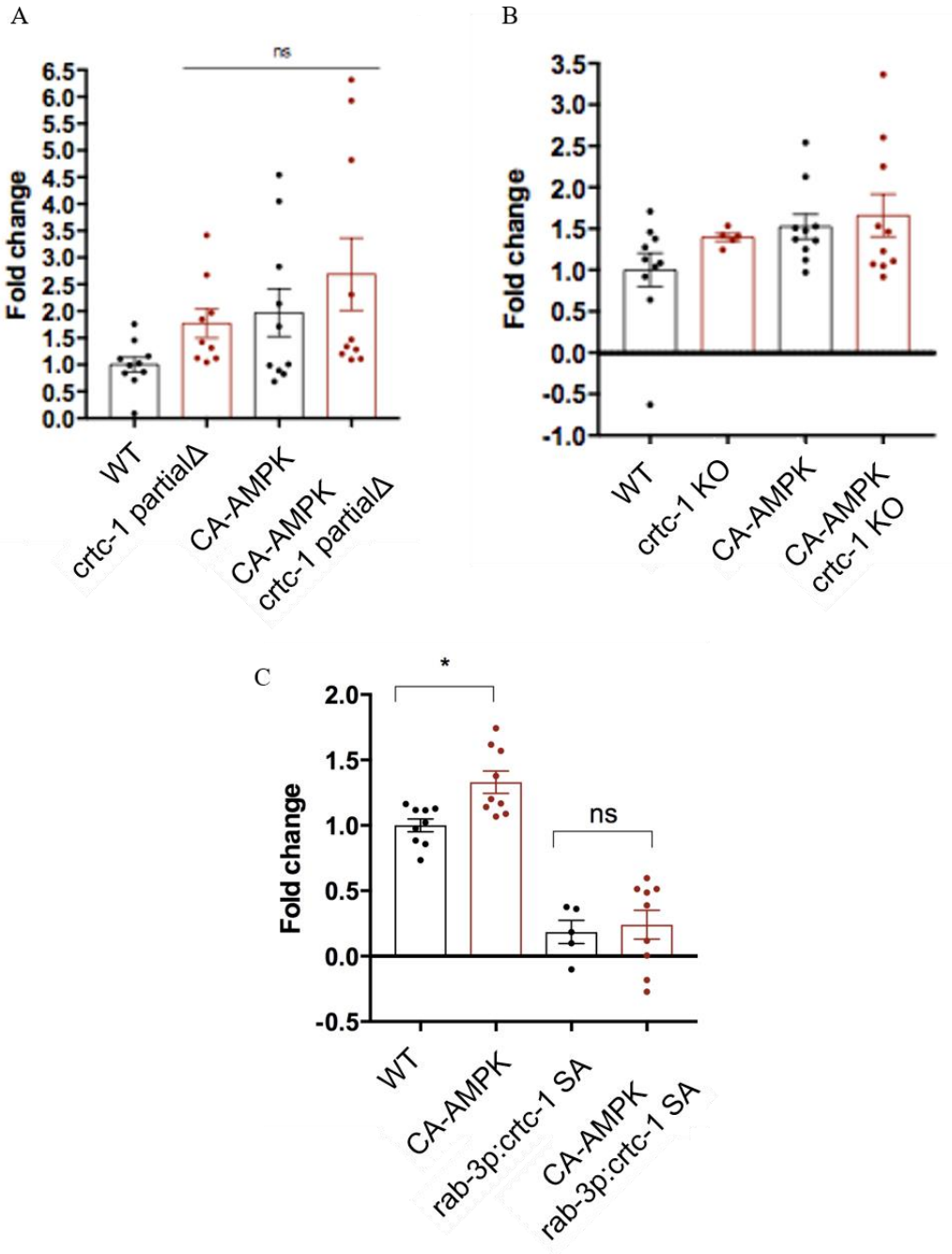

Figure 5.4: Neuronal CRTC-1 S>A blocks AMPK mediated behavioral plasticity

A) Memory assay performed at day1 on WT vs. *crtc-1* partialΔ (*crtc-1*^{tm2869}) vs. CA-AMPK vs. CA-AMPK x *crtc-1* partialΔ. Means: (WT) 1±0.14, (*crtc-1* partialΔ) 1.77±0.27, (CA-AMPK) 1.96±0.44, (CA-AMPK x *crtc-1* partialΔ) 2.68±0.67. One way ANOVA WT vs. *all other conditions* $p<0.05$, (CA-AMPK) vs (*crtc-1* partialΔ) vs. (CA-AMPK x *crtc-1* partialΔ) p=ns

2 independent biological replicates.

B) Memory assay performed at day1 on WT vs. *crtc-1*$^{-/-}$ (CRISPR knock out strain) vs. CA-AMPK vs. CA-AMPK x *crtc-1*$^{-/-}$. Means: (WT) 1±0.10, (*crtc-1*$^{-/-}$) 1.29±0.07, (CA-AMPK) 1.29±0.09, (CA-AMPK x *crtc-1*$^{-/-}$) 1.39±0.15. One way ANOVA WT vs. *all other conditions* $p<0.05$, (CA-AMPK) vs (*crtc-1*$^{-/-}$) vs. (CA-AMPK x *crtc-1*$^{-/-}$) p=ns. 2 independent biological replicates.
C) Memory assay performed at day1 on WT vs. CA-AMPK vs. *rab-3p:crtc-1* S>A vs. CA-AMPK x *rab-3p:crtc-1* S>A worms. Means: (WT) 1±0.04, (CA-AMPK) 1.33±0.08, (*rab-3p:crtc-1* SA) 0.18±0.08. (CA-AMPK x *rab-3p:crtc-1* SA) 0.24±0.11. t-test WT vs. CA-AMPK p=0.0039, *rab-3p:crtc-1* S>A vs. CA-AMPK x *rab-3p:crtc-1* S>A p=0.7374. 2 biological replicates.

5.3. Discussion

Taken together these results suggest that CRTC-1 inhibition acts as a downstream mediator of AMPK in the nervous system to regulate neuronal plasticity. Given that nuclear exclusion of CRTC-1 in the sensory neurons is sufficient to impair behavioral plasticity, we should test whether it would also be sufficient to block AMPK mediated effects. Nuclear CRTC-1 has been shown in mammalian systems to enhance synaptic transmission by driving a CREB dependent transcriptional response[300,315,316]. This contrasts with our data in which *crtc-1* inhibition enhances neuronal functions. This could be explained by the fact that our short-term learning and memory assay protocol does not rely on CREB. Indeed, *crh-1* (*C. elegans* CREB homolog) did not impair behavioral response in this assay. This is not completely surprising given that CREB function is more important for long term memory formation[330]. The effect we are seeing of CRTC-1 might

therefore not be mediated by CREB, which could explain the discrepancy with the mammalian data. The CRTCs bind to other transcription factors such as AFT6 or CBP[1], and it is possible that in *C. elegans*, *crtc-1* mediates its effects on neuronal plasticity through interaction with another transcription factor. Finding the downstream effector of CRTC-1 in *C. elegans* neurons mediating this response would give us more insight into how loss of function of *crtc-1* can be beneficial. Additionally, it is possible that CRTC-1 mediates its effects in neurons via a non-transcriptional function. The loss of function alleles that we used (*crtc-1* KO and *crtc-1* partialΔ) both affect the transactivation domain of the protein but also the splicing domain. We therefore cannot completely exclude a contribution of this domain in our experiments. With CRISPR technology now being widely used, it would be interesting to generated domain specific deletions of CRTC-1 protein to dissect their relative contribution to our phenotype. Generating a *crtc-1* point mutant unable to bind to CREB would allow us to examine the requirement of CREB binding for CRTC-1 mediated behavioral effects.

The phenotype we observed could be caused by a systemic effect of neuronal CRTC-1 expression. Indeed, the requirement of CRTC-1 inhibition for AMPK beneficial on behavioral plasticity effect mimics the longevity phenotype in *C. elegans*[296] and mirrors the detrimental impact of CRTC hyperactivation observed in metabolic disease and cancer[1]. *Burkewitz et al.* demonstrated that neuronal CRTC-1 can cell non-autonomously regulate peripheral metabolism and more specifically mitochondrial fragmentation via a catecholamine signal[331]. It is therefore possible that expression of CRTC-1 S>A in all neurons or in the sensory neurons might regulate a peripheral response which can feedback on neurons to modulate behavioral response. We previously demonstrated the requirement of mitochondrial fusion specifically in the neurons for AMPK enhanced behavioral plasticity. We should therefore explore whether modulation of CRTC-1 activity in the neurons regulates mitochondrial morphology cell autonomously and whether mitochondrial fusion is also required downstream of CRTC-1.

Finally, in order to reconcile the opposing effects of CRTC-1 on neuronal death and behavioral plasticity, we aim to examine the role of AMPK on neurodegeneration. It is unclear whether

AMPK activation is beneficial or detrimental in the context of neurodegeneration, with evidence pointing towards a potential deleterious activation of AMPK induced by $A\beta^{1-42}$ accumulation[156]. It is also unclear how protein aggregation leads to neuronal death even though mechanisms like calcium dysregulation[332] or ER stress are thought to contribute to it. Neuronal death probably involves different cellular processes than the ones mediating learning and memory formation. It is therefore possible that AMPK and CRTC-1 have different effects on these outcomes, which should be studied separately.

6. Chapter VI Modulation of pre-mRNA splicing as a downstream mediator of DR

6.1. Introduction

As we discussed in the introduction, an alternative approach to the management and treatment of age-related disorders is to understand and target age-related defects in cellular homeostasis that occur in the elderly. One potent intervention for modulating physiological age in model systems is Dietary Restriction (DR) or "reduction of food intake without malnutrition". DR elicits a physiological "low energy" state which prolongs lifespan and slows the rate of aging in species ranging from yeast to primates[3]. Beyond its effect on longevity, DR has a systemic effect on health, as it confers protection against age-related disorders such as neurodegenerative, cardiovascular and metabolic diseases and forms of cancers[38]. With the goal of developing novel therapeutics, the aging field has identified multiple downstream mediators of DR that might be targeted to mimic its beneficial effects on pathology. Among them two critical nutrient sensing enzymes, AMP activated protein kinase (AMPK) and target of rapamycin (TOR), which are antagonistically regulated by nutrient load in the cell. Activation of AMPK or suppression or TOR directly increase lifespan from yeast to mammals[77,79,80,82,333,334]. However, multiple cellular processes such as

translation, autophagy, metabolic homeostasis are regulated by both AMPK and TOR, and it remains unclear which of these homeostatic pathways link AMPK/TOR to healthy aging. The aim of the project I collaborated on was therefore to define cellular homeostatic mechanisms causally linking DR, AMPK and TOR to healthy aging, in order to determine novel therapeutic targets.

Aging is characterized by the loss of cellular and organismal homeostasis through an accumulation of cellular damage both at the level of the genome, with decline in transcriptional homeostasis and at the level of the proteome with a loss of proteostasis[335-337]. However, an underexplored homeostatic process required for cellular function is splicing of pre-mRNAs, a critical intermediary step in the central dogma. Alternative pre-mRNA splicing is a key link between DNA and protein, allowing for increased complexity in higher organisms through the generation of multiple transcripts from one coding sequence in the genome[338]. Deregulation of the splicing machinery has been strongly implicated in multiple age-related chronic diseases including neurodegeneration, leading to aberrant pre-mRNA processing and dysfunction[339,340]. These various changes in the splicing machinery may create vulnerabilities in cells, making them more

prone to develop a disease or less resistant to external insults. Aging is also associated with defects in RNA homeostasis and processing. Increased alternative splicing events are seen during normal ageing in multiple tissues in mouse[341], and observations of alternatively spliced variants specific to certain types or subtypes of cancers[342] have raised the hypothesis that RNA splicing can be directly linked to cancer progression. However, it remains unclear whether age-onset defects RNA homeostasis might play a role in the aging process and induce a permissive state for disease progression in old age.

During my PhD work in Dr. William Mair's laboratory, Dr. Caroline Heintz conducted a study on the mechanisms linking aging, DR and splicing homeostasis mediated longevity in order to gain insight into the aging process and provide novel targets for age related diseases treatment[343]. Dr. Heintz established pre-mRNA splicing as a novel mediator of DR induced longevity in *C. elegans* and demonstrated that modulation of specific spliceosome components rather than the overall splicing process promoted healthy aging, likely through remodeling of metabolism. Working with Dr. Heintz, I reproduced some of the keys findings of her publication. The data presented here are

published as part of Dr. Heintz first author publication[343], in which I am a contributing author. (All the data presented in this chapter was performed by myself unless otherwise noted).

6.2. Results

Previous data showed that an *in vivo* splicing reporter strain expressing a pair of *ret-1* exon 5 reporter minigenes with differential frame shifts could be used to study alternative splicing in the worm[343,344]. *Heintz et al*. confirmed that deregulation of the splicing reporter correlates with loss of splicing fidelity *in vivo* and can be used as a non-interventional surrogate readout of globalized defects in RNA processing. Using this reporter, previous data in the lab showed a progressive dysregulation of the splicing pattern with age, indicating aberrant RNA processing and dysregulation of alternative splicing in old worms[343]. If alternative splicing and splicing fidelity is causal to aging, blocking the effects of DR on splicing should inhibit DR longevity. Dr. Heintz performed a targeted reverse genetic screen for spliceosome components by comparing WT and DR longevity of animals subjected to RNAi of splicing factors using the *eat-2 (ad1116)* mutant strain as a genetic model for DR. As expected, given the critical role of splicing in organismal

homeostasis, inhibition of core conserved components of the spliceosome like *hrp-2* and *snr-1* significantly reduced lifespan of both WT and DR animals (**Fig6.1C,D**).

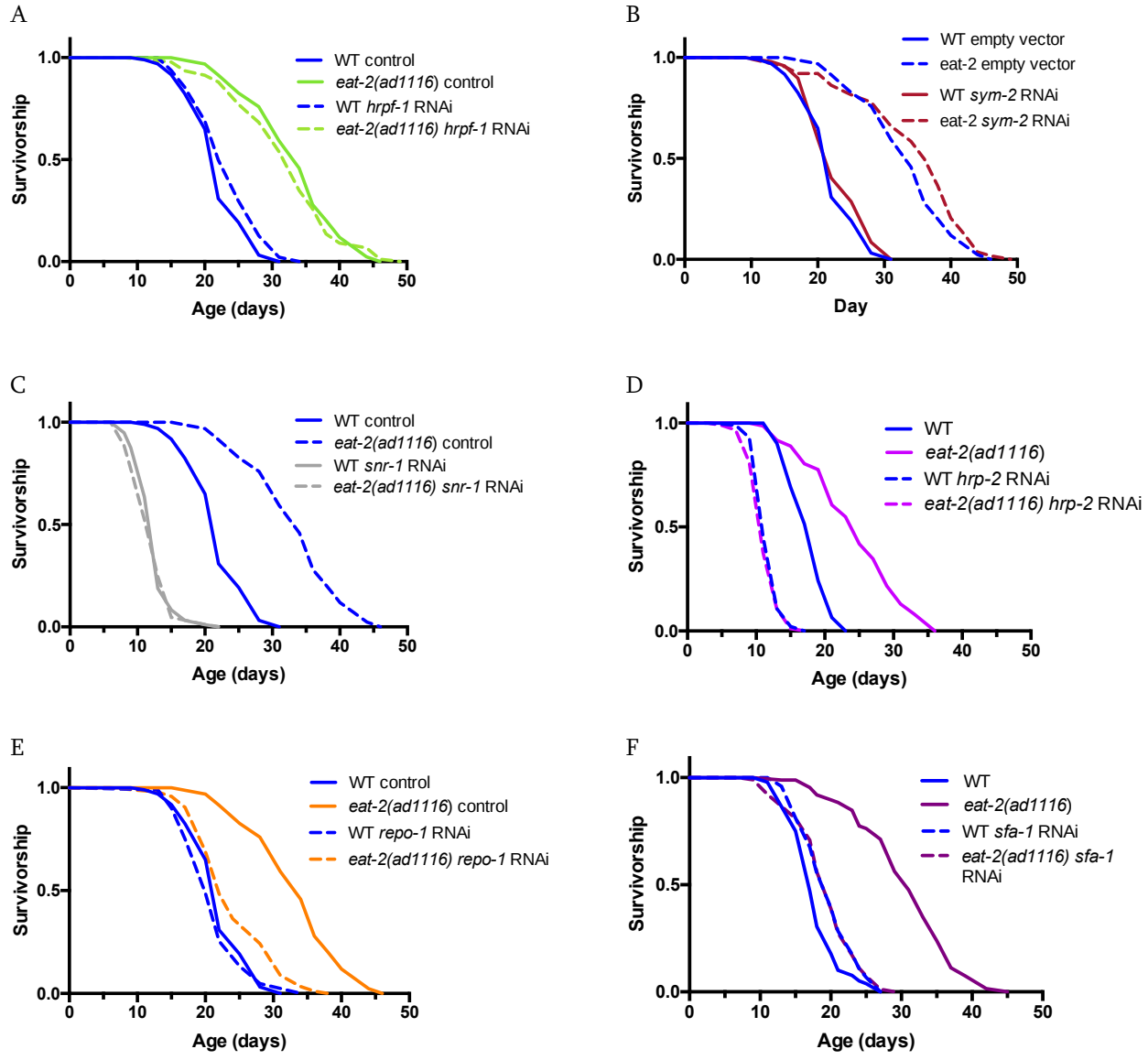

Figure 6.1: Specific splicing factors are required for DR mediated longevity

A) Representative lifespan curve of WT vs. *eat-2*$^{-/-}$ worms fed HT115 bacteria expressing control/empty vector vs. *hrpf-1* RNAi. Worms were scored as described in *Materials&Methods*. Control (WT vs. *eat-2*$^{-/-}$) $p<0.05$, *hrpf-1* RNAi (WT vs. *eat-2*$^{-/-}$) $p<0.05$

B) Representative lifespan curve of WT vs. *eat-2*$^{-/-}$ worms fed HT115 bacteria expressing control/empty vector vs. *sym-2* RNAi. Worms were scored as described in *Materials&Methods*. Empty vector (WT vs. *eat-2*$^{-/-}$) $p<0.05$, *sym-2* RNAi (WT vs. *eat-2*$^{-/-}$) $p<0.05$

C) Representative lifespan curve of WT vs. *eat-2*$^{-/-}$ worms fed HT115 bacteria expressing control/empty vector vs. *snr-1* RNAi. Worms were scored as described in *Materials&Methods*. Control (WT vs. *eat-2*$^{-/-}$) $p<0.05$, *snr-1* RNAi (WT vs. *eat-2*$^{-/-}$) p= ns

D) Representative lifespan curve of WT vs. *eat-2*$^{-/-}$ worms fed HT115 bacteria expressing control/empty vector vs. *hrp-2* RNAi. Worms were scored as described in *Materials&Methods*. Control (WT vs. *eat-2*$^{-/-}$) $p<0.05$, *hrp-2* RNAi (WT vs. *eat-2*$^{-/-}$) p= ns

E) Representative lifespan curve of WT vs. *eat-2*$^{-/-}$ worms fed HT115 bacteria expressing control/empty vector vs. *repo-1* RNAi. Worms were scored as described in *Materials&Methods*. Control (WT vs. *eat-2*$^{-/-}$) $p<0.05$, *repo-1* RNAi (WT vs. *eat-2*$^{-/-}$) p= ns

F) Representative lifespan curve of WT vs. *eat-2*$^{-/-}$ worms fed HT115 bacteria expressing control/empty vector vs. *sfa-1* RNAi. Worms were scored as described in *Materials&Methods*. Control (WT vs. *eat-2*$^{-/-}$) $p<0.05$, *sfa-1* RNAi (WT vs. *eat-2*$^{-/-}$) p= ns

Despite blocking DR longevity, such detrimental effects on lifespan do not prove a causal role for splicing homeostasis in DR longevity. The inability of *eat-2* mutation to extend lifespan of the very short-lived *snr*-1 and *hrp*-2 knocked down strains could merely be due to an overall sickness of the nematodes and an inability to respond to any stimuli. Knock down of other factors like *hrpf-1* and *sym-2* did not affect WT or DR lifespan, suggesting that remodeling of splicing dynamics does not necessarily impair lifespan **(Fig6.1A,B)**. More importantly and supporting a causal role for altered spliceosome dynamics in effects on DR, the knock down of two splicing

factors, REPO-1 and SFA-1 blocked the lifespan extension in the DR model without having adverse effects on WT lifespan **(Fig6.1E,F)**. While *repo*-1 RNAi partially blocked DR lifespan, *sfa-1* RNAi completely abolished DR mediated longevity. These lifespans demonstrate the specificity of the different spliceosome components. Indeed, knock down of various components of the spliceosome, a central cellular process, does not necessarily lead to shortened lifespan, suggesting a functional redundancy among these proteins. Although the knock down of multiple splicing factors blocked *eat-2* deletion mediated lifespan extension (**Fig6.1C-F**), we were not interested in studying splicing factors with a detrimental effect on WT. Their effect on DR mediated longevity might only reflect overall damage to the animal's physiology rather than specificity in mediating the response to DR. The branch point binding protein SFA-1 (splicing factor 1) therefore represented a more promising target as its effect was specific to DR mediated longevity (**Fig6.1F**).

Given the requirement of SFA-1 for *eat-2*$^{-/-}$ mediated longevity, a genetic model of DR, we asked whether this effect was conserved in other longevity models, such as TOR and AMPK,

known downstream mediators of DR. Direct suppression of an upstream activator of TORC1, RAGA1, increases lifespan and TORC1 is implicated as a mediator of the beneficial effects of DR[345]. However, the mechanisms by which TORC1 links DR to lifespan are unclear[37]. Previous reports have linked TORC1 to regulation of splicing[346-351], we therefore asked whether there was a direct role of RNA splicing in lifespan extension mediated by TORC1 suppression. As shown by others[351], suppression of *raga-1* (TOR Complex 1 activator Rag GTPase), the upstream regulator of TORC1 which senses amino acid levels, induces robust lifespan extension. Strikingly, *sfa-1* RNAi knock down fully suppressed the longevity of *raga-1 (ok386)* mutants **(Fig6.2A)**. Furthermore, SFA-1 was also required for the lifespan extension by loss of the TORC1 downstream target S6 Kinase/RSKS-1 **(Fig6.2B)**. Taken together, these results suggest that the role of SFA-1 extends beyond RAGA-1 as a general regulator of TORC1 mediated longevity and highlight the role of splicing efficiency and SFA-1 downstream of TORC1. Given that not all splicing factors recapitulate the effects of SFA-1, we hypothesized that SFA-1 had a more targeted role than overall RNA splicing homeostasis and might modulate specific cellular processes to mediated longevity. To test this hypothesis, we inhibited a key RNA homeostatic mechanism, the

non-sense mediated decay (NMD) pathway, using a temperature sensitive mutant (*smg-1* gene carries a point mutation which makes the protein unstable and degraded at higher temperatures, ie. 24 Celsius) and showed that the NMD pathway is not required for *raga-1* RNAi mediated lifespan extension (**Fig6.2C**). To determine whether SFA-1 modulates the effects of DR on metabolism, we tested the role of SFA-1 in AMPK induced lifespan extension, a conserved regulator of metabolic homeostasis, activated by low energy and required for some DR longevity in *C. elegans*[351]. Overexpression of the catalytic subunit of AMPK, AAK-2, extends lifespan[296] and this effect is suppressed by *sfa-1* RNAi (**Fig6.2D**). SFA-1 is therefore required in multiple longevity models in *C. elegans* directly modulating metabolism. The additional observation that blocking the NMD pathway, a key RNA homeostatic process, did not recapitulate knock down of *sfa-1* effect prompted us to ask what the specific genes or cellular functions altered by SFA-1 mediating longevity were.

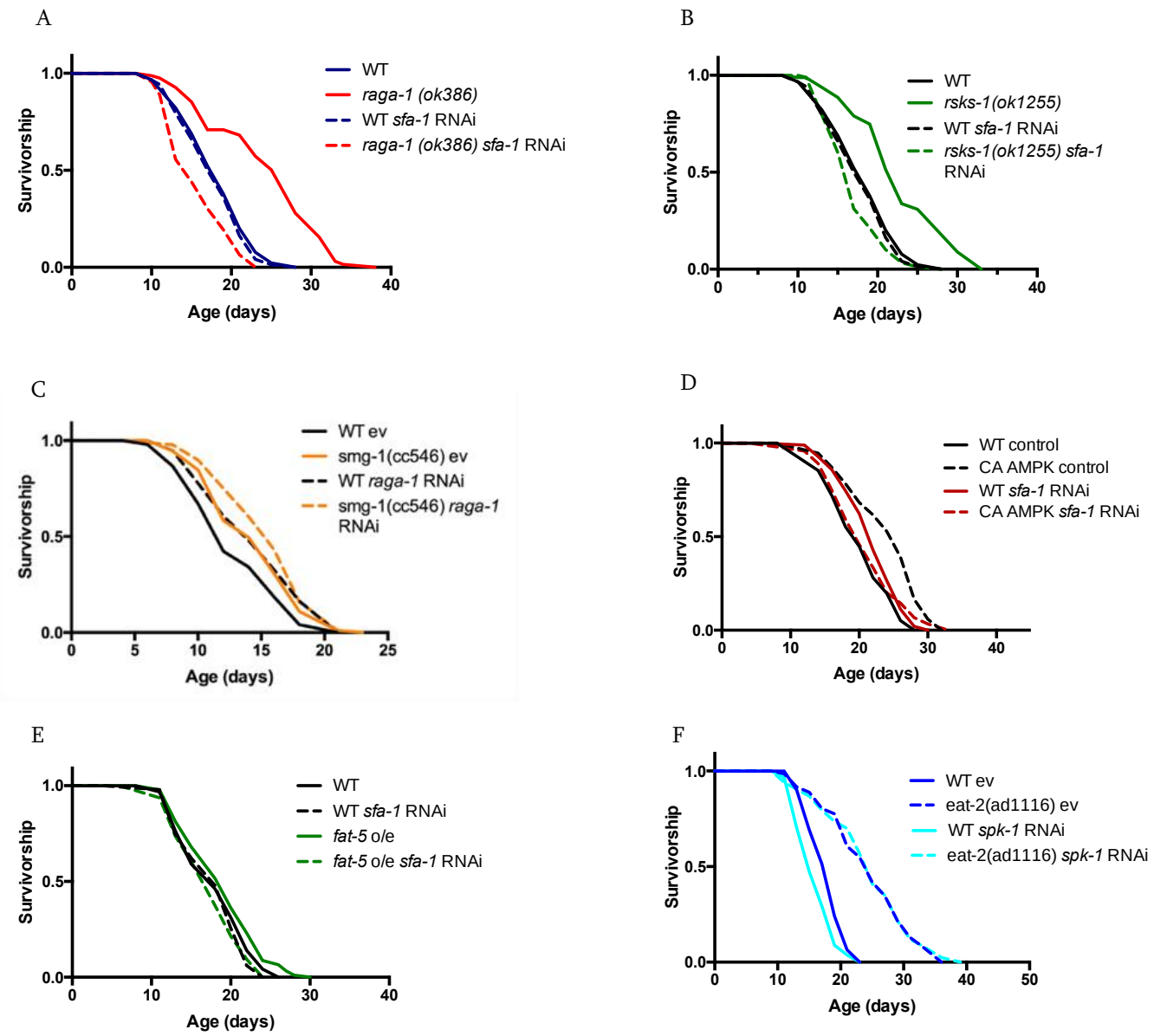

Figure 6.2: SFA-1 is required for AMPK and TOR mediated longevity in *C. elegans*

A) Representative lifespan curve of WT vs. *raga-1*$^{-/-}$ worms fed HT115 bacteria expressing control/empty vector vs. *sfa-1* RNAi. Worms were scored as described in *Materials&Methods*. Control (WT vs. *raga-1*$^{-/-}$) $p<0.05$, *sfa-1* RNAi (WT vs. *eat-2*$^{-/-}$) p= ns

B) Representative lifespan curve of WT vs. *rsks-1*$^{-/-}$ worms fed HT115 bacteria expressing control/empty vector vs. *sfa-1* RNAi. Worms were scored as described in *Materials&Methods*. Empty vector (WT vs. *rsks-1*$^{-/-}$) $p<0.05$, *sfa-1* RNAi (WT vs. *eat-2*$^{-/-}$) p= ns

C) Representative lifespan curve of WT vs. *smg-1*^{ts} worms fed HT115 bacteria expressing control/empty vector vs. *raga-1* RNAi. Worms were scored as described in *Materials&Methods*. Control (WT vs. *smg-1*^{ts}) $p<0.05$, *raga-1* RNAi (WT vs. *smg-1*^{ts}) $p<0.05$

D) Representative lifespan curve of WT vs. CA-AMPKworms fed HT115 bacteria expressing control/empty vector vs. *sfa-1* RNAi. Worms were scored as described in *Materials&Methods*. Control (WT vs. CA-AMPK) $p<0.05$, *sfa-1* RNAi (WT vs. CA-AMPK) p= ns

E) Representative lifespan curve of WT vs. *fat-5O/E* worms fed HT115 bacteria expressing control/empty vector vs. *sfa-1* RNAi. Worms were scored as described in *Materials&Methods*. Control (WT vs. *fat-5O/E*) p= ns, *sfa-1* RNAi (WT vs. *fat-5O/E*) p= ns

F) Representative lifespan curve of WT vs. *eat-2*$^{-/-}$ worms fed HT115 bacteria expressing control/empty vector vs. *spk-1* RNAi. Worms were scored as described in *Materials&Methods*. Control (WT vs. *eat-2*$^{-/-}$) $p<0.05$, *spk-1* RNAi (WT vs. *eat-2*$^{-/-}$) $p<0.05$

By analyzing a previous RNAseq performed in our lab on young versus old animals fed *ad libitum* or dietary restricted and +/- *sfa-1* RNAi, we observed that *sfa-1* RNAi did not affect all DR related gene expression changes but specifically reversed the up regulation of lipid and fatty acid metabolism genes, including *fat-5* gene. We asked whether overexpression of *fat-5* would be sufficient to extend lifespan and whether this effect required *sfa-1* **(Fig6.2E)**. Unfortunately, we observed no significant difference between the treatments, suggesting that modulating fatty acid metabolism through *fat-5* is not sufficient to recapitulate DR mediated longevity.

Finally, we aimed at understanding how the activity of SFA-1 itself is regulated as the RNAseq analysis revealed no difference in gene expression with age. We hypothesized that SFA-1 activity might be regulated through phosphorylation. To test this hypothesis, we asked whether knock down of a putative SFA-1 kinase, *spk-1*, was required for DR mediated longevity **(Fig6.2F)**. We observed no significant difference between the treatments. Further work is thus needed to understand the post-translational regulation of SFA-1 during aging. Together, these data define SFA-1 as a novel modulator not only of DR and TOR/AMPK longevity but also of the effects of DR on metabolism and homeostasis.

6.3. Discussion

The study from *Heintz et al*. in which I participated established RNA splicing as a key mediator of the aging process and pre-mRNA splicing homeostasis can be used as a biomarker and predictor of life expectancy in the model organism *C. elegans*. Aging induces global spliceosome disruption as characterized by intron retention or unannotated splice junctions, indicative of aberrant transcripts which can be reduced by DR in an SFA-1 dependent manner. SFA-1 not only blocks

DR mediated longevity but is also required for lifespan extension by modulation of some if its downstream components, AMPK and TOR via RAGA-1 and RSKS-1. Furthermore, Dr. Heintz showed that *sfa-1* overexpression was sufficient for lifespan extension. Although evidence suggest that SFA-1 potentially mediates its effects through modulation of cellular metabolism such as lipid and fatty acid oxidation or mitochondrial respiration, the exact mechanisms remain to be elucidated. These findings open a new field in the research in aging and given the evidence from the literature that RNA splicing dysregulation can lead to disease progression[339,340], it would be interesting to ask if DR also requires spliceosome function and in particular SFA-1 to mediate its beneficial effects on the onset of age related disorders. Recently, splicing deficiency has been suggested as a potential contributor to TDP-43 (Transactive response DNA binding Protein 43 kDa) induced proteinopathy, a model for two neurodegenerative disorders, Fronto Temporal Dementia (FTD) and Amyotrophic Lateral Sclerosis (ALS). TDP-43 normally represses the splicing of non-conserved cryptic exons thus maintaining intron integrity but this process is impaired in ALS-FTD patients[352]. We showed here a very specific role for SFA-1 in promoting

healthy aging and it would be interesting to define whether other spliceosome components play a similarly specific role in the onset of age-related pathologies such as neurodegeneration.

7. Chapter VIII Conclusion: significance and future directions

7.1. Conclusion

The dramatic increase in life expectancy during the 20th century was accompanied by a resultant epidemic of age-related pathologies including neurodegenerative diseases such as AD. Unfortunately, current therapeutics primarily focusing on protein misfolding aspects of AD have been unsuccessful in the clinical trials. The only available treatments for patients suffering from AD are symptomatic drugs targeting the cholinergic system[353] and these drugs do not impact disease progression. As an example, Bapineuzumab, the first monoclonal antibody targeted against Aβ peptide failed in phase III clinical trials as it did not provide significant clinical benefit despite reducing Aβ levels in the brain[354,355]. Recent epidemiological studies have suggested a strong association between metabolic dysfunction and neurodegeneration[34]. Therefore, an alternative approach is to target metabolic pathways disrupted in AD models for therapeutics[35]. AMP activated protein kinase (AMPK) is activated in a low energy state via sensing the AMP:ATP ratio. Once active, AMPK promotes longevity in model organisms such as *C. elegans*, and protects against a wide range of age related diseases including AD[76]. In addition, AMPK regulates

mitochondrial homeostasis and mitochondrial networks in mammals[294], a process dysregulated in the context of AD. However, whether mitochondrial regulation causally links AMPK to protection against neurodegeneration is unknown. Here we used a learning and memory protocol in *C. elegans* as readout of neuronal function[254]. We showed that nematodes expressing the toxic amyloid peptide $A\beta^{1-42}$ in the neurons display impaired learning ability, which can be rescued by constitutive activation of AMPK (CA-AMPK). We further showed that CA-AMPK enhances learning ability in wild type nematodes by promoting mitochondrial fusion in the neurons. Indeed, fusion deficient worms show impaired learning, which could be rescued by restoring mitochondrial fusion specifically in the neurons. Additional findings suggest that AMPK might promote its beneficial effects on neuronal function via inhibition of CREB-regulated transcriptional co-activator 1 (CRTC-1). We showed that *crtc-1* deletion nematodes displayed improved behavioral plasticity whereas CRTC-1 constitutive nuclear localization in the nervous system impaired neuronal functions in wild type animals. Blocking the link between AMPK and CRTC-1 by mutation of AMPK phosphorylation sites blocked the enhanced learning capacity induced by AMPK activation. My thesis work provides more understanding in the role of AMPK in

neurodegeneration and highlight a novel link between AMPK activation and modulation of mitochondrial fusion in the nervous system to promote behavioral plasticity. More work will be needed to understand which proteins in this process can be pharmacologically targeted to benefit patients.

7.2. Using *C. elegans* as a model for cognitive decline

We chose the model system *C. elegans* for our study for the availability of genetic tools and the ease to generate transgenic animals. The transparency of the nematodes also conferred a distinct advantage as we were able to image mitochondrial morphology *in vivo*. Furthermore, *C. elegans* carries numerous homologs of the mammalian genomes, making it a relevant model system. We were able to explore the link between AMPK and mitochondrial morphology or other potential downstream effectors such as CRTC-1, interactions which are conserved in mammals. One major difference between the nematode nervous system and the mammalian brain, which could have impaired the testing of our working hypothesis, is the fact that *C. elegans* is a post mitotic organism, meaning that neurogenesis does not occur in the adult worm. Adult Hippocampal Neurogenesis, or the production and integration of new neurons in the brain, which mainly occurs

in the dentate gyrus in mammals[356], has been linked to learning and memory performance in both aversive association paradigms[357-359] and positive association paradigm[360] and is considered to have a crucial role in the hippocampal learning process. Although we cannot assess the relative contribution of adult neurogenesis to learning capacity in our system, we still observed a plasticity of the behavior in *C. elegans* after conditioning, suggesting that we are studying the intra-cellular processes involved in regulation of behavioral plasticity. Nevertheless, in spite of being a powerful aging model, very amenable for experimental manipulations, *C. elegans* cannot model all aspects of neurodegenerative disorders pathophysiology. Brain inflammation is thought to be a key driver of several neurodegenerative diseases pathogenesis such as AD, one aspect being abnormal microglial activation[32,33]. Unfortunately, glial cells in *C. elegans* do not recapitulate the function of mammalian microglia thus limiting the use of *C. elegans* in testing its contribution. *C. elegans* represents a valuable tool to study neurobiology and gain insight into the cellular and molecular processes disrupted in age related disorders however, it will be necessary to validate those findings not only in rodents but in more complex organisms such as primates or humans[223].

7.3. Targeting AMPK

AMPK plays an important role in neurons physiology in mammals (**cf. Introduction**) and evidence in mice suggest a beneficial role of AMPK activation in memory formation. However, whether AMPK might benefit or impair cognitive outcome in neurodegenerative diseases remains unclear. On one side, studies have suggested that AMPK can improve pathogenesis through modulation of synaptic function and long-term depression (cf. Introduction). On the other side, AMPK might be abnormally activated in AD and contribute to disease progression by acting as a tau kinase. Many studies use $A\beta^{1-42}$ accumulation as a readout of the efficacy of the tested intervention and although Aβ peptide accumulation is a hallmark of AD, it does not always reflect the severity of the disease. We therefore used the measurement of neuronal functions, more specifically behavioral plasticity, as our main outcome and we showed a beneficial role of AMPK activation. Our findings do not exclude a potential detrimental role of AMPK activation as we could not evaluate the effect of AMPK modulation on tau pathology in our system. Several *C. elegans* models expressing pathological levels of tau in the neurons are available and it would be interesting to test whether AMPK activation can benefit those models also through modulation of

mitochondrial dynamics. Because AMPK downstream mediators are numerous and act in very diverse pathways [cf intro], it is also possible that AMPK has antagonistic effects in AD models. We should therefore understand the exact downstream mechanisms mediating AMPK function in the nervous system. In our model, we showed enhanced behavioral plasticity and complete rescue of $A\beta^{1-42}$ induced deficits by systemic activation of AMPK. Although we further showed that this effect was mediated by mitochondrial fusion specifically in the neurons we cannot exclude a systemic contribution of AMPK activation. Using CRIPR-Cas9 technology, we could tissue specifically express a single copy of either WT or activated form of AMPK in order to explore the tissue requirement of AMPK itself. Even though the brain seems to be the tissue of interest to target, a systemic action of AMPK activation could be very interesting when considering translational potential as crossing the blood brain barrier in humans represents one of the main problematics in developing drugs for neurological disorders. Although the AMPK activator, metformin, has been shown to cross the blood brain barrier in mice, metformin was delivered systemically in all studies to the animals and also improved metabolic outcomes, such as insulin resistance and glucose tolerance.

7.4. Other downstream mediator of AMPK contributing to beneficial effect

Here we defined mitochondrial fusion as a required downstream mechanism for AMPK mediated beneficial effect on cognitive function in the context of aging and neurodegeneration. As we discussed earlier, mitochondrial biology is key to neuronal physiology and has been linked to cognitive disorders and is therefore a relevant target to study downstream of AMPK. However, AMPK is a master regulator of metabolism in the cell and regulates a vast array of processes, which might contribute to the effect of AMPK modulation in the context of neurodegeneration. AMPK regulate autophagy by interacting with ULK1/2 and Beclin-1 and this catabolic response to starvation serves one purpose, the recycling of cytosolic content such as the organelles[125]. Autophagy would be a relevant downstream mechanism to study here as it plays a role in physiological degradation of the amyloid peptides contained in endosomes in the brain[11]. Autophagy is also dysregulated in AD as mutation of the Prenisilin 1 gene disrupts autophagy by inducing a loss of lysosomal acidification[361]. Deletion of Beclin-1, a downstream target of AMPK, leads to increased Aβ accumulation in AD mice models while its overexpression alleviates the proteotoxic accumulation in cultured neurons[362,363]. Inducing autophagy therefore represents a

physiological way to degrade the accumulated aggregates and slow the progression of the disease. mTOR signaling is a potent upstream inhibitor of autophagy and the mTOR inhibitor drug, Rapamycin, was found to decrease Aβ accumulation in cells[364] and improve the cognitive functions in AD mice models[364,365]. *Caccamo et al*., however, do not demonstrate the requirement of autophagy for Rapamycin mediated improvement of mice response in the memory task[364]. Although activation of autophagy could be a downstream mediator of Rapamycin, mTOR signaling modulates multiple other cellular processes, for example through AMPK inhibition, which could possibly mediate its beneficial effects. Teasing out the exact mechanism of action of drugs like Rapamycin is labor intensive but much needed as a more specific mechanism of action would provide a better therapeutic target. The relative contribution and the relevance of autophagy as a target in neurodegenerative disorders such as AD remains to be further explored.

8. Supplemental Publications

Heintz, C. *et al.* Splicing factor 1 modulates dietary restriction and TORC1 pathway longevity in *C. elegans*. *Nature* **541**, 102-106, doi:10.1038/nature20789 (2017).

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