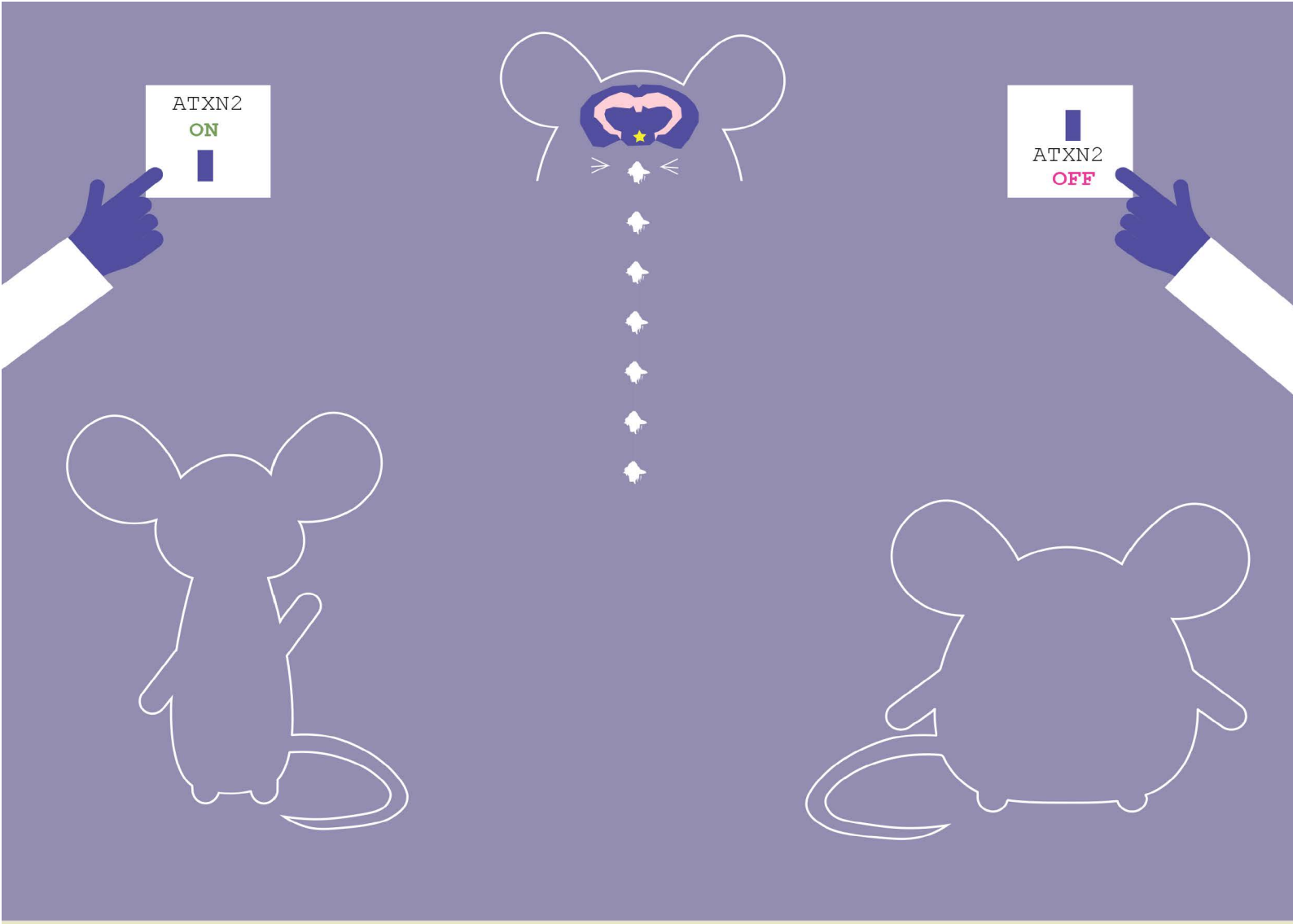

Sara Matias Carmo Silva

UNRAVELLING THE ROLE OF ATAXIN-2 IN THE HYPOTHALAMUS: A NEW PLAYER IN METABOLISM

Tese de doutoramento em Ciências Farmacêuticas, na especialidade de Biologia Celular e Molecular, orientada pela Professora Doutora Cláudia Margarida Gonçalves Cavadas e pelo Professor Doutor Luís Fernando Morgado Pereira de Almeida, apresentada à Faculdade de Farmácia da Universidade de Coimbra.

Dezembro 2016

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Universidade de Coimbra

Unravelling the role of ataxin-2 in the hypothalamus: a new player in metabolism

Investigar o papel da ataxina-2 no hipotálamo: um novo mediador metabólico

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FFUC FACULDADE DE FARMÁCIA
UNIVERSIDADE DE COIMBRA

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Sara Matias Carmo Silva

Dissertation presented to Faculty of Pharmacy of the University of Coimbra in fulfillment of the requirements for a Doctoral degree in Pharmaceutical Sciences, speciality in Cellular and Molecular Biology.

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Cover note: Hypothalamic ataxin-2 modulation effect on body weight regulation. Ataxin-2 silencing in the hypothalamus promotes weight gain and ataxin-2 overexpression prevents weight gain upon high fat diet feeding. Illustration kindly provided by George Foot and José Seco.

“Words are, in my not-so-humble opinion,

our most inexhaustible source of magic”

J.K. Rowling, *Harry Potter and the Deathly Hallows*

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Table of Contents

List of abbreviations

4E-BP Eukaryotic initiation factor 4E-binding protein

A2BP1 Ataxin-2 binding protein

A2D Ataxin-2 domain protein

A2RP Ataxin-2 related protein

ACADS Acyl-CoA dehydrogenase, C-2 to C-3 short chain

ACTH Adrenocorticotropic hormone

Ago1 Argonaute 1

AgRP Agouti-Related Protein

AHN Anterior hypothalamic nucleus

Akt Protein kinase B

ALS Amyotrophic Lateral Sclerosis

AMPA α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

AMPK 5' AMP-activated protein kinase

ANS Autonomic nervous system

APO A-1 Apolipoprotein A-1

ARH Arcuate nucleus of the hypothalamus

ARNTL1/ BMAL1 Aryl hydrocarbon receptor nuclear translocator like

Atp2a2 ATPase sarcoplasmic/endoplasmic reticulum Ca^{2+} transporting 2

ATX-2 *C. elegans* ortholog of ATXN2

ATXN2 Ataxin-2

ATXN2L Ataxin-2 like protein

AVP Arginine vasopressin

Aβ Amyloid beta peptide

BAC Bacterial artificial chromosome

BAT Brown adipose tissue

BBB Blood-brain barrier

BCA Bicinchoninic acid

BCAA Branched chain amino acid

BMI Body mass index

BSA Bovine serum albumin

CAA Cytosine-Adenine-Adenine

CAG Cytosine-Adenine-Guanine

CART Cocaine-and-amphetamine-regulated-transcript

cDNA complementary Deoxyribonucleic acid

CLOCK Circadian locomotor output cycles kaput

CNS Central nervous system

CRH Corticotropin releasing hormone

Cry Cryptochrome

CSF Cerebrospinal fluid

dAtx2 *Drosophila* homolog of ATXN2

DDX6 DEAD box helicase 6

DMH Dorsomedial nucleus

DNA Deoxyribonucleic acid

DR Dietary restriction

DTT Dithiothreitol

ECF Enhanced chemiofluorescence

EGFR Epidermal growth factor receptor

eIF4G eukaryotic initiation factor 4G

ER Endoplasmic reticulum

ERK5 Extracellular-signal-regulated kinase 5

FAS Fatty acid synthase

FMR1 Fragil X mental retardation protein

FSH Follicle-stimulating

FTO Fat mass and obesity associated

FUS Fused in sarcoma protein

G3BP1 G3BP stress granule assembly factor 1

GFAP Glial fibrillary acidic protein

GFP Green fluorescent protein

GH Growth hormone

GHRS1 Ghrelin receptor

GM3 Monosialodihexosylganglioside

GnRH Gonadotropin-releasing hormone

GWAS Genome-wide association study

HD Huntington's disease

HDL High-density lipoprotein

Het Heterozigous

HFD High fat diet

HGP Hepatic glucose production

HIF1α Hypoxia-inducible factor 1-alpha

HPA Hypothalamic-pituitary-adrenocortical axis

HPG Hypothalamic– pituitary–gonadal axis

ICV Intracerebroventricular

IGF-1 Insulin-like growth factor 1

IL-1β Interleukine 1-Beta

Inpp5a Inositol polyphosphate-5-phosphatase a

Insig1 Insulin-induced gene 1

InsR Insulin receptor

IP(3)R1 Type 1 inositol (1,4,5)-triphosphate receptor

IRS1 Insulin receptor substrate 1

ITPR1 Inositol 1,4,5-Trisphosphate Receptor Type 1

ITT Insulin tolerance test

JNK c-Jun N-terminal kinase

kITT Glucose clearance rate

KO Knockout

Kog1 Ubiquitin-binding TORC1 subunit

LD Linkage disequilibrium

LepR Leptin receptor

LH Luteinizing hormones

LHA Lateral hypothalamic area

LN Local interneuron

Lsm Like-Sm domain

LsmAD Like-Sm associated domain

MBH Mediobasal hypothalamus

MC4R Melanocortin receptor 4

MDA Malondialdehyde

Me31B Maternal expression at 31B

MEF Mouse embryonic fibroblasts

miRISC MicroRNA-induced silencing complex

miRNA microRNA

MJD Machado-Joseph disease

mRNA messenger Ribonucleic acid

mTOR Mammalian target of rampamycin

Mttp Microsomal triglyceride transfer protein

NAFLD Non-alcoholic fatty liver disease

NF-κB Nuclear factor kappa-light-chain-enhancer of activated B cells

NMDAR1 N-methyl-D-aspartate receptor

NPY Neuropeptide Y

Otp Orthopedia

P-bodies processing bodies

Pab1p Poly(A) Binding protein 1

PABP Polyadenylate (poly(A))-binding protein

PAM2 Polyadenylate (poly(A))-binding protein interacting motif

PAR-5 14-3-3-like protein 1

Pbp1 Pab1p-binding protein 1

PBS Phosphate buffer saline

PER Period

PKC Protein kinase C

Plin3 Perilipin-3

PMSF Phenyl- methylsulphonylfluoride

PN Projection neuron

POA Preoptic area

PolyA Polyadenosine sequence

PolyQ Polyglutamine tract

POMC Pro-OpioMelanocortin

PON Postoptic nucleus

PPARδ Peroxisome proliferator-activated receptor gamma

PSP Progressive Supranuclear Palsy

PVDF Polyvinylidene fluoride

PVN Paraventricular nucleus

RBM9 RNA-binding motif protein 9

RBPMS RNA-binding protein with multiple splicing

REM Rapid eye movement

RIPA Radio-immunoprecipitation assay-buffer

RIPA Radio-immunoprecipitation assay-buffer

RISC RNA-induced silencing complex

RNA Ribonucleic acid

RNAi RNA interference

RNP Ribonucleoprotein particle

Rora Retinoic-related orphan receptor A

ROS Reactive oxygen species

rpm rotations per minute

RPS6 Ribosomal protein S6

RRM RNA recognition motifs

RTK Receptor tyrosine kinases

SBMA Spinal bulbar muscular atrophy

SCA Spinocerebellar ataxia

SCA1 Spinocerebellar ataxia type 1

SCA2 Spinocerebellar ataxia type 2

SCN Suprachiasmatic nucleus

SDS-PAGE Sodium dodecyl sulphate-polyacrylamide

Sh Short hairpin

SH2 SRC Homology 2

SH2B3 SH2B Adaptor Protein 3

SH3 SRC Homology 3

SK Small conductance calcium channels

SNP Single nucleotide polymorphism

SNS Sympathetic nervous system

SOCS3 Suppressor of cytokine signaling 3

TBS Tris-buffered saline

TDP-43 TAR DNA-binding protein 43

TG Triglycerides

TGF-β1 Transforming growth factor beta-1

TNF-α Tumor necrosis factor alpha

Tor1 Phosphatidylinositol kinase-related protein kinase

TORC1 mTOR complex 1

TORC2 mTOR complex 2

TRP Transient receptor potential channels

tyf twenty-four gene

UTR Untranslated region

VDCC Voltage-dependent calcium channels

VLDL Very-low-density lipoproteins

VLH Ventrolateral hypothalamus

VMH Ventromedial nucleus

WAT White adipose tissue

WT Wild-type

ZBRK1 BRCA1-interacting protein with a KRAB domain 1

ZEN-4 Kinesin-like protein

ZNF350 Zinc-finger protein 350

α-MSH Alpha-melanocyte stimulating hormone

Resumo

O hipotálamo desempenha diversas funções fisiológicas, nomeadamente como sensor do estado nutricional e regulador metabólico. O hipotálamo, como regulador do apetite, é ainda relevante no desenvolvimento de alterações metabólicas como a obesidade e resistência à insulina. Por outro lado, a obesidade altera a fisiologia do hipotálamo. Assim, o estudo de novos mecanismos envolvidos na função do hipotálamo no controlo da homeostase metabólica, mais especificamente no núcleo arqueado, pode contribuir para a descoberta de novas estratégias terapêuticas para o tratamento da resistência à insulina ou da obesidade.

A ataxina-2 é uma proteína ubíqua que está presente no hipotálamo e que está envolvida em diversos mecanismos celulares como o metabolismo do RNA, resposta celular ao stress, endocitose e organização do citoesqueleto. Trabalhos anteriores mostraram que murganhos "knockout" para a ataxina-2 eram obesos, apresentando dislipidémia e resistência à insulina. Adicionalmente, a ataxina-2 está envolvida em várias funções fisiológicas controladas pelo hipotálamo, como o ritmo circadiano e resposta ao stress. No entanto, até há data, o papel da ataxina-2 no hipotálamo nunca foi investigado.

O primeiro objetivo deste trabalho foi estudar o papel da ataxina-2 no hipotálamo na regulação do apetite, peso corporal e homeostase energética em murganhos. Para tal, o gene da ataxina-2 foi especificamente silenciando, no núcleo arqueado do hipotálamo de murganhos C57BL/6, recorrendo a uma injeção esterotáxica com vetores lentivirais que codificam para um short-hairpin que tem como alvo a ataxina-2. Os resultados mostram que os animais com silenciamento da ataxina-2 no núcleo arqueado consomem mais comida (hiperfagia), são obesos e resistentes à insulina. Além disso, apresentaram marcadores de inflamação no hipotálamo (TNF-α, IL-1β, TGF1β, GFAP e SOCS3), acumulação de lípidos no fígado, aumento da quantidade de tecido adiposo branco e aumento de gotas lipídicas no tecido adiposo castanho. Os resultados obtidos mostram que o silenciamento da ataxina-2 no hipotálamo tem efeitos semelhantes a uma dieta rica em lípidos.

Por outro lado, murganhos alimentados durante 4 semanas com uma ração rica em lípidos (45% gordura) têm menor quantidade de ataxina-2 no hipotálamo, em comparação a murganhos mantidos em ração normal. Assim, o segundo objetivo deste trabalho foi investigar se o restabelecimento dos níveis de ataxina-2 no hipotálamo nos murganhos alimentados com ração rica em lípidos poderia melhorar ou diminuir o estado de obesidade e resistência à insulina. Os níveis de ataxina-2 no hipotálamo de murganhos foram aumentados

utilizando vetores lentivirais que codificam para a ataxina-2. O restabelecimento dos níveis de ataxina-2 no núcleo arqueado do hipotálamo preveniu a obesidade causada pela dieta rica em lípidos e melhorou a sensibilidade à insulina, diminuiu os marcadores de inflamação no hipotálamo e diminuiu a acumulação de lípidos no fígado, tecido adiposo branco e castanho.

Outros estudos mostraram que os murganhos "knockout" para a ataxina-2 são resistentes à insulina. Assim, o terceiro objetivo deste trabalho consistiu em avaliar se o restabelecimento especifico dos níveis de ataxina-2 no hipotálamo dos animais "knockout" para a ataxina-2 promoveria um aumento na sensibilidade à insulina. Utilizando a mesma estratégia referida anteriormente, os níveis de ataxina-2 foram especificamente restabelecidos no núcleo arqueado do hipotálamo de murganhos "knockout" para a ataxina-2. Os resultados obtidos mostraram que a expressão hipotalâmica de ataxina-2 diminuiu a resistência à insulina destes animais. Adicionalmente, observamos os murganhos "knockout" para a ataxina-2 apresentaram perturbações no comportamento circadiano e alterações na expressão de genes relógio (Bmal1, CLOCK e Per2) no hipotálamo. O aumento da expressão de ataxina-2 no hipotálamo restaurou os níveis de expressão dos genes relógio.

Com o objetivo de avaliar o papel da ataxina-2 na sinalização da insulina, modulamos os níveis de ataxina-2 *in vitro* numa linha celular de neurónios hipotalâmicos de murganho (mHypo-N42) num paradigma de resistência à insulina. Observamos uma diminuição nos níveis de ataxina-2 e um aumento em fatores da via metabólica do mTOR, neste modelo de resistência à insulina induzida pelo palmitato. Adicionalmente, a sobrexepressão de ataxina-2 promoveu a diminuição da ativação de mediadores da via do mTOR, o que traduz uma reversão nos efeitos da resistência à insulina.

Em conclusão, os resultados desta tese mostram que a ataxina-2 tem um papel relevante na regulação hipotalâmica do metabolismo. O impacto da ataxina-2 no peso corporal e na homeostase metabólica sugerem que o gene ATXN2 poderá estar relacionado com obesidade. Considerando que a obesidade diminui os níveis de ataxina-2 no hipotálamo, o que promove disfunção metabólica, propomos que o restabelecimento dos níveis de ataxina-2 como uma possível abordagem terapêutica para a obesidade e consequentes alterações metabólicas.

Palavras chave: Ataxina-2, Hipotálamo, Obesidade, Inflamação hipotalâmica, Insulina, mTOR, Ritmo circadiano.

Abstract

The hypothalamus has several physiological functions, namely as a sensor of the whole-body nutritional status and metabolic regulator. The hypothalamus, as an appetite regulator, plays a relevant role in the development of metabolic alterations, such as obesity and insulin resistance. On the other hand, obesity changes hypothalamic physiology. For this reason, understanding the mechanisms of hypothalamic function, more specifically of the arcuate nucleus, might contribute for the discovery of new therapeutic approaches for the treatment of insulin resistance or obesity.

Ataxin-2 is an ubiquitous protein that is present in the hypothalamus and it has relevant role in several cellular mechanisms, such as RNA metabolism, cellular stress response, endocytosis and cytoskeleton organization. Previous work showed that ataxin-2 knockout mice are obese, have dyslipidaemia and insulin resistance. Moreover, ataxin-2 is involved in several physiological functions regulated by the hypothalamus, such as circadian rhythm and stress response. However, to this date, the role of ataxin-2 in the hypothalamus was never investigated.

The first aim of this work was to unravel the role of ataxin-2 in the hypothalamus in the regulation of appetite, body weight and energy homeostasis in mice. For this purpose, we specifically silenced ataxin-2 in the arcuate nucleus of the hypothalamus of C57BL/6 mice, by stereotaxic injection of lentiviral vectors encoding for a short-hairpin targeting ataxin-2. The results show that mice with ataxin-2 silencing in the arcuate nucleus consume more food (hyperphagia), and are obese and insulin resistant. Furthermore, these mice have higher levels of inflammation and gliosis markers (TNF-α, IL-1β, TGF1β, GFAP e SOCS3 in the hypothalamus), have lipid accumulation in the liver, increased white adipose tissue content and larger lipid droplets in brown adipose tissue. These results suggest that silencing ataxin-2 in the hypothalamus has similar effects as high fat diet (HFD).

Mice fed with a HFD (45% fat) for 4 weeks have lower levels of ataxin-2 in the hypothalamus, when compared to mice fed with a chow diet. Therefore, the second aim of this work was to investigate whether the reestablishment of ataxin-2 in the hypothalamus could improve or decrease the obesity and insulin resistance induced by HFD. In order to specifically increase ataxin-2 levels in the hypothalamus, we stereotaxically injected lentiviral vectors encoding for ataxin-2 in mice arcuate nucleus. This reestablishment of ataxin-2 in hypothalamic arcuate nucleus prevented HFD-induced obesity and improved insulin sensitivity, reduced

inflammatory markers in the hypothalamus and decreased lipid accumulation in the liver, white and brown adipose tissue.

Previous studies showed that ataxin-2 knockout mice are insulin resistant. Therefore, the third aim of this work was to evaluate if the specific reestablishment of ataxin-2 in the hypothalamus of ataxin-2 knockout mice could improve insulin sensitivity. Using the same approach as previously described, we restored ataxin-2 expression in the arcuate nucleus of the hypothalamus of ataxin-2 knockout mice. The results showed that ataxin-2 knockout mice with reestablishment of ataxin-2 in the hypothalamus have improved insulin sensitivity. Furthermore, we observed that the ataxin-2 knockout mice have dysfunction of circadian behaviour and altered levels of clock genes (Bmal1, CLOCK e Per2) in the hypothalamus. The reestablishment of ataxin-2 in the hypothalamus of these mice restored clock genes expression.

To evaluate the role of ataxin-2 in insulin signalling, we modulated ataxin-2 levels *in vitro* in a cellular model of mouse hypothalamic neurons (mHypo-N42) in an insulin resistance paradigm induced by palmitate. We observed a decrease in ataxin-2 and a overactivation of the mTOR pathway in this model of insulin resistance. Additionally, the overexpression of ataxin-2 decreased the activation of mTOR signalling pathway effectors, which is related to an amelioration of insulin resistance deleterious effects.

Overall, the results of this thesis show that ataxin-2 has a relevant role in hypothalamic mediated metabolic regulation. Ataxin-2 changed body weight and metabolic homeostasis suggesting ATXN2 gene as a potential obesity gene. Since obesity decreases ataxin-2 levels and this promotes metabolic dysfunction, we propose the reestablishment of ataxin-2 levels as a potential therapeutic approach for obesity and related metabolic disorders.

Keywords: Ataxin-2, Hypothalamus, Obesity, Hypothalamic inflammation, Insulin, mTOR, Circadian rhythm

CHAPTER 1

General Introduction

1.1. Ataxin-2

Ataxin-2 is a polyglutamine protein with different cellular functions, ranging from RNA metabolism to cytoskeleton reorganization (*Magaña et al., 2013*).

The gene encoding this protein was localized in chromosome 12q23 – 12q24.1 in 1993 and gained the denomination of *SCA2* gene since it was discovered in the context of Spinocerebellar ataxia type 2 (SCA2) (*Gispert et al., 1993*). SCA2 is an autosomal dominant neurodegenerative disease, caused by a mutation in *SCA2* gene (*Pulst et al., 1996; Sanpei et al., 1996, Imbert et al., 1996*). It was only in 1996 that an abnormal expansion of CAG trinucleotide in exon 1 of the *SCA2* gene was identified as causative of the disease and ataxin-2 identified as the gene product (*Pulst et al., 1996; Sanpei et al., 1996, Imbert et al., 1996*). Since then, both *SCA2* and *ATXN2* denominations are used for the gene.

1.1.1. ATXN2 gene

The *SCA2,* or *ATXN2,* gene contains 25 exons with approximately 130Kb of genomic DNA (*Sahba et al., 1998*) (Figure 1). At exon 1, *ATXN2* gene encodes an unstable triplet repeat expansion (CAG) that codifies for a glutamine-rich stretch in the N-terminal region of the protein. In normal individual alleles of *ATXN2* gene carry 13-31 CAG-repeats, while the presence of more than 31 repeats leads to neurodegeneration (*Pulst et al., 1996; Sanpei et al., 1996, Imbert et al., 1996; Pulst et al., 2005*). However, this range of repeats has relatively low mutation rate and is stably inherited. The genetic stability of normal repeats is thought to be achieved by the presence of CAA interruptions between the CAG repetition, reading (CAG)8-CAA-$(CAG)_4$-CAA-$(CAG)_8$. Normal CAG repeat tracts have usually one or two interruptions (*Andrés et al., 2003; Ramos et al., 2010*). Other reports show interruptions in CAG repeats of intermediate-expanded alleles (27-33) (*Yu et al., 2011; Conforti et al., 2013*). However, some authors report that expanded alleles of ATXN2 are uninterrupted, hence the instability of this mutated alleles upon inheritance, and the tendency to further expand (*Choudhry et al., 2001; Sobczak and Krzyzosiak, 2005*).

ATXN2 gene was also correlated with several other disorders (Table 1), namely Amyotrophic lateral sclerosis (ALS), where intermediate-size expansions of the CAG tract act as a risk factor for the age of onset of motor neurons degeneration and manifestation of the disease (*Elden et al., 2010; Chen et al., 2011; Soraru et al., 2011; Daoud et al., 2011*) and even for ALS patient survival (*Chió et al., 2015; Borghero et al., 2015*).

ATXN2 intermediate expansions and a polymorphism in this gene were also correlated with the age of onset of another neurodegenerative disorder, Machado-Joseph Disease (MJD)/ Spinocerebellar ataxia type 3 (MJD) (*Tezenas et al., 2014; Ding et al., 2016*). Additionally, it was shown that ATXN2 product, ataxin-2, is decreased in MJD patients and animal models and its re-establishment can attenuate neuronal degeneration caused by mutant ataxin-3 (*Lessing and Bonini, 2008; Nóbrega et al., 2015*).

Table 1. Ataxin-2 contribution to diseases different of SCA2

Disease	*Model*	*Phenotype*	*Reference*
SCA1	*Fly*	Ataxin-2 upregulation enhances mutant-ataxin-1 toxicity	*Al-Rahami et al., 2007*
MJD	*Fly*	Ataxin-2 upregulation enhances mutant-ataxin-3 toxicity	*Lessing and Bonini, 2008*
	Mouse	Ataxin-2 upregulation rescues motor phenotype and decreases mutant ataxin-3 levels	*Nobrega et al., 2015*
	Humans	Hypermethylation of ATXN2 leads to earlier onset	*Laffita-Mesa et al., 2012*
		ATXN2 CAG expansions promote earlier onset	*Tezenas et al., 2014; Chen et al., 2016*
		ATXN2 SNP rs7969300 promotes earlier onset	*Ding et al., 2016*
ALS	*Yeast* *Fly* *Mammalian cells*	Ataxin-2 with intermediate CAG repeat enhances mutant TDP-43 toxicity	*Elden et al., 2010*
	Humans	Intermediate CAG repeats in ATXN2 correlate with increased risk for development of the disease	*Daoud et al., 2011; Laffita-Mesa et al., 2013; Neuenschwander et al., 2014*
		Cerebellar loss in patients with ATXN2 with intermediate CAG tract expansion	*Tan et al., 2016*
		Longer CAG repeats shorten survival	*Chió et al., 2015; Borghero et al., 2015*
ALS-FTD	*Humans*	Intermediate CAG repeats in ATXN2 correlate with increased risk for development of the disease	*Bäumer et al., 2014; Sellier et al., 2016; Ciura et al., 2016*
Parkinson's Disease and Parkinsonism	*Humans*	CAG repeats promote disturbances in ATXN2-mediated RNA metabolism observed in the disease	*Nkiliza et al., 2016; Sen at al., 2016*
		CAG repeats in ATXN2 correlate with prevalence of the disease	*Kim et al., 2007; Yamashita et al., 2014*
Schizophrenia	*Humans*	ATXN2 SNP rs7969300 confers vulnerability to disease	*Zhang et al., 2014; Davies et al., 2016*
Cancer	*Mammalian Cells* *Humans*	Ataxin-2 promotes apoptosis in neuroblastoma cells Tumour cells more sensitive to treatment (chemotherapy) present higher ataxin-2 levels	*Wiedemeyer et al., 2003*

1.1.2. Ataxin-2 structure

Ataxin-2 is composed of 1312 amino acid residues (including the mean of 22 glutamines of the polyQ stretch), and has an approximated molecular mass of 140 kDa (*Pulst et al., 1996; Sanpei et al., 1996, Imbert et al., 1996*). Except for the polyglutamine domain in its N-terminal, ataxin-2 exhibits no similarity with other polyglutamine-rich proteins associated with neurological diseases. The N-terminal domain increases ataxin-2 molecular mass according to the length of the CAG-repeat expansion (*Sahba et al., 1998; Albrecht et al., 2004; reviwed in Magaña et al., 2013*) (Figure 1).

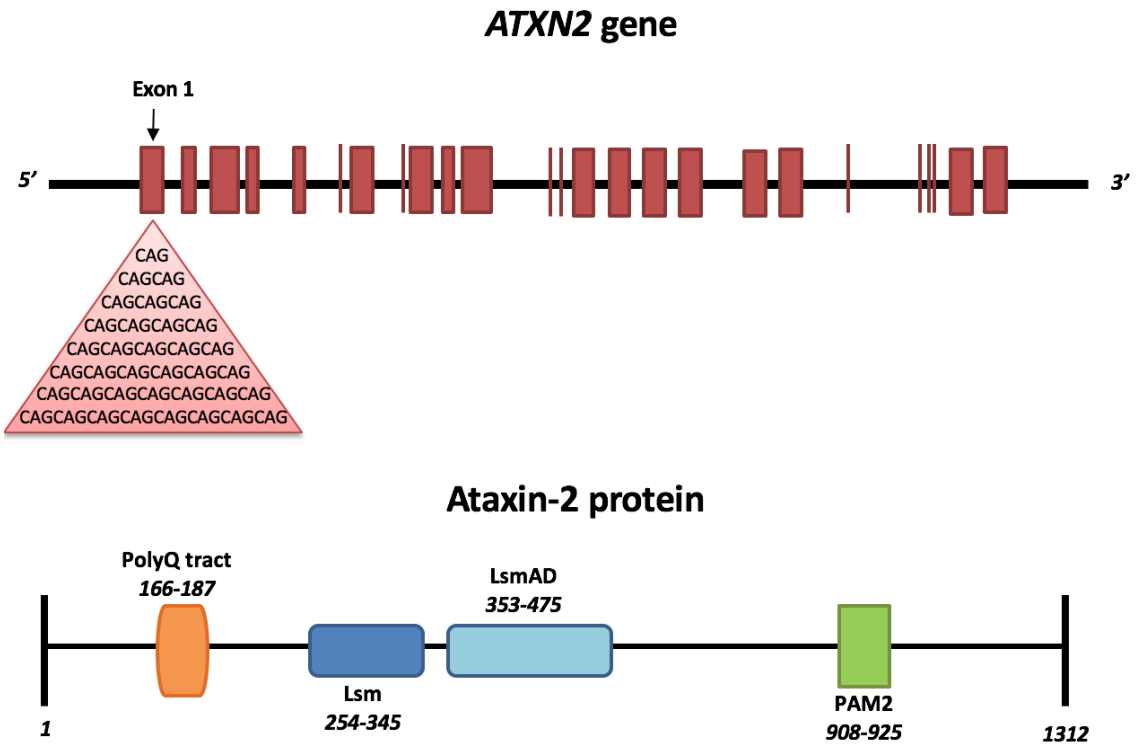

Figure 1. A. Structure of the ATXN2 gene human ataxin-2. Gene localized in the human chromosome 12. Wide and narrow boxes represent the 25 exons encoding ataxin-2 protein. A number superior to 31 CAG repeats in exon 1 produces the clinical manifestations of SCA2. **B. Structure of ataxin-2**. The PolyQ tract in the N-terminal region, the two globular domains, Lsm and LsmAD domains, and the PAM2 motif in the C-terminal region. The protein is constituted by 1312 amino acids. Lsm: Like Sm; LsmAD: Like Sm-associated domain; PAM2: poly(A)-binding protein interacting motif; PolyQ: Polyglutamine stretch. Adapted from (Magaña et al., 2013)

Ataxin-2 is a highly basic protein with an isoelectric point of 9.6 due to the elevated content in glutamine residues. The acidic region of 456 amino acids covers roughly exons 2-7 and is predicted to have two globular domains named Lsm (Like Sm, amino acid 254-345) and LsmAD (Lsm- associated domain, amino acid 353-475) (Figure 1) (*Neuwald et al., 1998; Albrecht et al., 2004; Satterfield and Pallanck, 2006; van de Loo et al., 2009; Yokoshi et al., 2014*). The Lsm

domain contains Sm1 and Sm2 motifs, which are found in proteins involved in RNA splicing, suggesting that ataxin-2 might function as a component of the RNA splicing complex. The LsmAD with mainly α-helices, contains both a clathrin-mediated trans-Golgi signal and an endoplasmic reticulum exit signal (*Neuwald et al., 1998; Shibata et al., 2000; Huynh et al., 2003; Albrecht et al., 2004; Satterfield and Pallanck, 2006*).

Additionally, ataxin-2 presents the non-globular N- and C-terminal tails (amino acid 1–253 and 476–1312). At the N-terminal is located the polyglutamine stretch (PolyQ) and at the C-terminal is located a short sequence motif called PAM2, that stands for poly(A)-binding protein (PABP) interacting motif (*Neuwald et al., 1998; Kozlov et al., 2001; Albrecht et al., 2004*). As the name suggests, this domain is responsible for the binding of PABP, a protein involved in RNA metabolism that mediates several ataxin-2 functions, specially mRNA translation (*Satterfield and Pallanck, 2006*; *van de Loo et al., 2009; Damrath et al., 2012; Yokoshi et al., 2014*).

1.1.3. Ataxin-2 distribution

Ataxin-2 has a widespread expression in adult and embryonic tissues, predominantly in the brain, but also in the heart, muscle, gut, liver and lung (*Imbert et al., 1996; Pulst et al., 1996; Nechiporuk et al., 1998; Huynh et al., 1999*). Ataxin-2 is present in neuronal cells of adult mouse hippocampus, thalamus and hypothalamus (*Huynh et al., 1999*). The higher levels of ataxin-2 are in cerebellar Purkinje cells, the primary site of SCA2 neurodegeneration (*Imbert et al., 1996; Pulst et al., 1996; Nechiporuk et al., 1998; Huyhn et al., 1999*). Ataxin-2 was also found in apical cytoplasm of pyramidal neurons of the frontal cortex, in the hippocampus, in the basal ganglia, globus pallidus, amygdala and *substantia nigra* (*Huynh et al., 1999*).

It was also observed moderate expression of ataxin-2 in the hypothalamus (*Huynh et al., 1999*). Interestingly, another study showed a strong expression of Ataxin-2 Binding Protein (A2BP1) also in the hypothalamus (*Hammock and Levitt, 2011*). The A2BP1 protein is an adaptor molecule of ataxin-2, which needs ataxin-2 to exert its functions (*Shibata et al., 2000*), supporting the presence of ataxin-2 in the hypothalamus.

Several studies investigated the subcellular localization of ataxin-2 and its main localization is in the cytoplasm (*Huynh et al., 2000*). Ataxin-2 is associated with polysomes, localized mainly at the rough endoplasmatic reticulum and at the Golgi complex (*Huynh et al., 2000; Huynh et al., 2003; Satterfield and Pallanck., 2006; Nonhoff et al., 2007; van de Loo et al., 2009; Hallen et al., 2011*).

Ataxin-2 is also localized in other cytoplasmic structures, such as stress granules and P-bodies (processing bodies) (*Nonhoff et al., 2007; Swisher and Parker, 2010*). These structures, formed upon cellular stress (from heat chock to nutrient privation), sequester mRNAs limiting protein synthesis during cellular disturbances (*Ralser et al., 2005a; Nonhoff et al., 2007; Heck et al., 2014*). It was also reported the presence of ataxin-2 in nuclear and plasma membranes (*Nonis et al. 2008; Hallen et al. 2011*). However, the mutated form of ataxin-2 can accumulate both in the cytoplasm and in the nucleus (*Huynh et al., 1999; Al Ramahii et al., 2007; Nobrega et al., 2015*).

Enhanced ataxin-2 levels have been observed in Purkinje cells of elderly people and in Purkinje cells of SCA2 patients, indicating that alterations of intracellular ataxin-2 may contribute to cellular dysfunction and could correlate with age and SCA2 progression (*Huynh et al., 1999; Koyano et al., 1999*). On the opposite, depletion of ataxin-2 was observed in the brain of MJD patients (*Nobrega et al., 2015*). However, interestingly, accumulation of ataxin-2 sensitizes neuroblastoma cells of young children for apoptosis, (*Wiedemeyer et al., 2003*). These observations suggest that ataxin-2 levels may oscillate in different conditions with different outcomes.

1.1.4. Ataxin-2 paralogs, homologues, orthologs and interacting partners

The ATXN2 gene is highly conserved throughout evolution (Figure 2). The most studied *ATXN2* homologs are *Atxn2* in *Mus musculus* (*Nechiporuk et al., 1998*), Pbp1 (Pa1b-binding protein) in *Saccharomyces cerevisiae* (*Mangus et al., 1998; Kozlov et al., 2001*) and *dAtx2* in *Drosophila melanogaster* (*Satterfield et al., 2002*); the ortholog, *ATX-2* in *Caenorhabditis elegans* (*Kiehl et al., 2000*). These homologues and ortholog have conserved Lsm and LsmAD domains (except for Drosophila that only presents a Lsm domain) as well as sequences that resemble the PAM2 motif, supporting a role in RNA metabolism (Figure 2). However, none of these genes presents codification for a polyglutamine tract. The mouse homolog however contains one glutamine at the site of the polyQ tract in the human gene (*Nechiporuck et al., 1998; Mangus et al., 1998; Kiehl et al., 2000; Kozlov et al., 2001; Satterfield et al., 2002; Huynh et al., 2003; Albrecht et al., 2004; Ralser et al., 2005a; Bravo et al., 2005*).

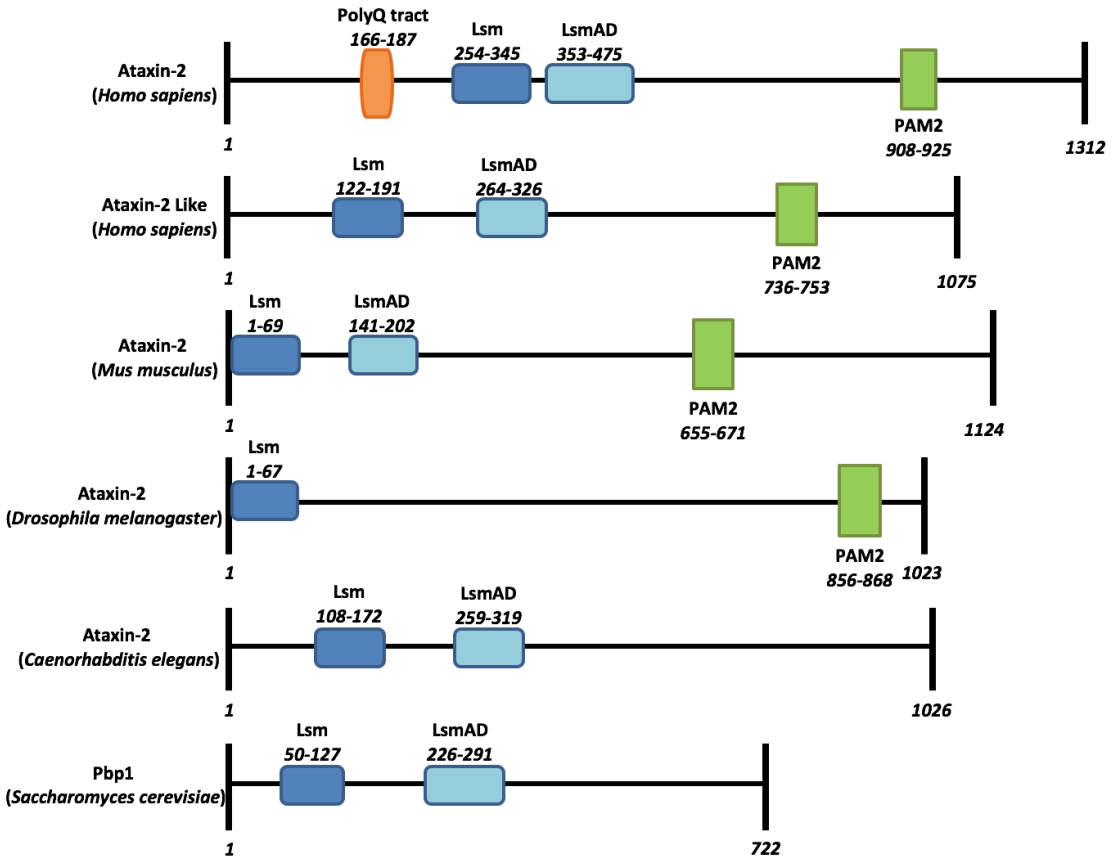

Figure 2. **Structures of *ATXN2* gene homologues and orthologs.** Domain architecture of human ataxin-2 protein and its paralog, homologues and ortholog. All the presented proteins shared conserved domains such as the Lsm, present in proteins involved in RNA metabolism. The PAM2 motif is not conserved in *C. elegans* and *S. cervesiae*, although these proteins also associate directly with PABP, in their structure do not present a conserved region for this specific binding. Only human ataxin-2 presents a PolyQ tract in its N-terminal. The structures presented help to understand the conserved role of ataxin-2 in different species. Based on: Satterfield et al., 2002; Albretch et al., 2005; Ralser et al., 2005; Protein sequences obtained from NCBI and UniProt databases (Database Resources of the National Center Biotechnology Information, 2016; UniProt: the universal protein knowledgebase, 2016); Homology between proteins was obtained using BLAST program (Database Resources of the National Center for Biotechnology Information, 2016).

Paralogs for ataxin-2 also have been studied in order to facilitate the understanding of molecular actions of this protein. Even though paralogs not always share the same functions, in the case of ATXN2, its paralogs have conserved functions. In 2003, a partial cDNA sequence in chromosome 16 showed high homology to the *ATXN2* gene and was named ataxin-2-related protein (A2RP) (*Figueroa and Pulst, 2003*). Another group identified a gene with homology to ATXN2 gene and identified it as ataxin-2 domain protein (A2D) (*Meunier et al., 2002*). Proteins of both families (A2RP and A2D) are usually referred as Ataxin-2 like (*ATXN2L*) (*Kaehler et al., 2012*). ATXN2L shows several conserved motifs namely the Lsm and LsmAD domains, the clathrin-mediated trans-Golgi signal, the endoplasmic reticulum (ER) exit signal and the PAM2

motif. The polyglutamine tract is not conserved between ATXN2 and ATXN2L (Figure 2) (*Kozlov et al., 2001; Meunier et al., 2002; Figueroa and Pulst, 2003*). ATXN2L is correlated with erythropoietin receptor and signalling (*Meunier et al., 2002*), a function not yet reported to ATXN2. However, ATXN2L is also associated with RNA metabolism and stress mRNA response regulation, through stress granules and P-bodies formation (*Kaehler et al., 2012*).

Ataxin-2 exerts a major part of its cellular functions through interactions with adaptor molecules, specially involved in RNA metabolism. PABPC1 (*Albrecht and Lengauer, 2004; Satterfield and Pallanck, 2006*) and A2BP1 (*Shibata et al., 2000*) are the main functional adaptors of ataxin-2 and through the study of these interactions it was possible to learn more about ATXN2 functions (*see next section for further detail*).

1.1.5. Functions of ataxin-2

Ataxin-2 interaction with proteins such as A2BP1 and PABPC1 was on the base of several studies regarding ataxin-2 function, which allowed inferring some of its functions. The family of proteins ATXN2L are also considered adaptors of ataxin-2 and may overlap some of the physiological functions of ataxin-2 (*Kozlov et al., 2001; Meunier et al., 2002; Figueroa and Pulst, 2003*). Paralogs such as ATXN2L can replace ataxin-2, which might also explain why the knockout of ataxin-2 is not lethal (*Kiehl et al., 2006*).

Ataxin-2 is involved in different cellular mechanisms such as transcription, RNA metabolism, translation regulation, stress granules formation, cytoskeleton reorganization, endocytosis, calcium homeostasis and other functions (Figure 3).

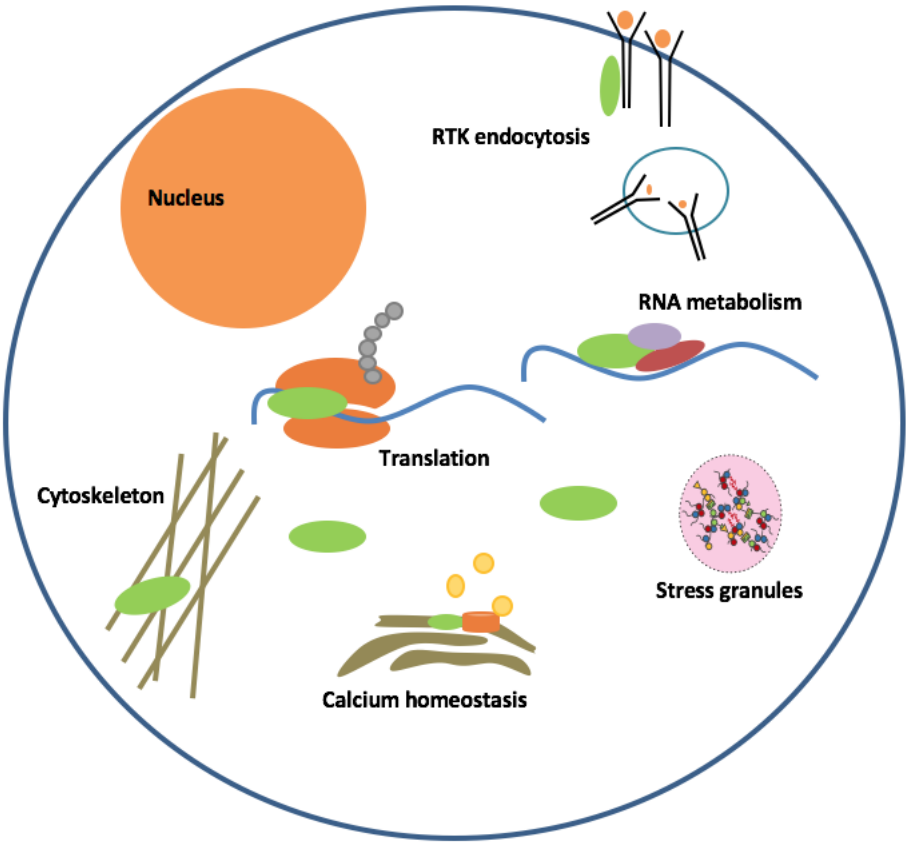

Figure 3. Participation of ataxin-2 in different cellular processes Ataxin-2 is represented as the green oval shape.

1.1.5.1. Ataxin-2 modulates transcription

There is one study that shows that ataxin-2 can act as a transcriptional modulator (*Hallen et al., 2011*). In this study, the authors describe that ataxin-2 directly binds to transcriptional regulator Zinc-finger protein 350 (ZNF350), also known as BRCA1-interacting protein with a KRAB domain 1 (ZBRK1). Ataxin-2 and ZBRK1 were found to form a complex and activate the transcription of the ATXN2 gene itself. Overexpression of ZBRK1 increased ataxin-2, whereas its depletion led to decreased ataxin-2 levels. This evidence suggests for the first time, that ataxin-2 can modulate transcription itself since it is able to control its own expression (*Hallen et al., 2011*).

1.1.5.2. Role of ataxin-2 on RNA metabolism

Ataxin-2 seems to be involved in mRNA stabilization, mRNA splicing and mRNA decay. Ataxin-2 is part of the Lsm protein family, presenting a Lsm and LsmAD domain (*Neuwald et al., 1998;*

Albrecht et al., 2004; Satterfield and Pallanck, 2006). Lsm proteins are involved in mRNA stability, splicing and decay and can bind directly to RNA and to other RNA splicing factors (*Wilusz and Wilusz, 2005; Tharun, 2009; Wilusz and Wilusz, 2013).* In fact, ataxin-2 was recently found to directly bind to the AU-rich elements (AREs) within the 3'UTR region of RNA (*Yokoshi et al., 2014*). By binding to the mRNA, ataxin-2 promotes protein translation through the stabilization of the mRNA. The stabilization of mRNA by ataxin-2 is in part mediated by PABPC1, since the deletion of PAM2 decreased mRNA stability (*Yokoshi et al., 2014).* The polyQ tract deletion also decreases mRNA stability, which might indicate that this region plays a determinant role in ataxin-2-mediated mRNA stability (*Yokoshi et al., 2014*).

One of the major interactor of ataxin-2 is PABPC1 that binds to its PAM2 domain (*Ralser et al., 2005a; Bravo et al., 2005; Kozlov et al., 2010*). PABPC1 participates in mRNA stability, splicing and decay, and it is a translation initiator (*Wang and Kiledjan, 2000; Peixeiro et al., 2012; Zhu et al., 2015*). In yeast, Pbp1 binds to Pab1p (the PABP homolog in yeast), to regulate mRNA polyadenylation (*Ralser et al., 2005a; Tharun, 2009*). It has been shown in a mouse model of SCA2, that PABPC1 is sequestered by triplet expansions, which might impair its binding to the 3' poly(A) tail (*Damrath et al., 2012*).

A2BP1 has ribonucleoprotein motifs, characteristic of RNA binding proteins, and binds to the carboxyl-terminal of ataxin-2 (*Shibata et al., 2000; Bravo et al., 2005*). A2BP1 is directly involved in RNA alternative splicing (*Underwood et al., 2005; Gehman et al., 2011*). Recently, it was demonstrated that A2BP1 regulates RNA splicing of the NMDAR1 gene receptor, which modulates excitatory synaptic transmission in the hippocampus, participating in long- term potentiation and learning (*Lee et al., 2009*). Ataxin-2 might modulate alternative splicing by binding directly to A2BP1, however no studies have demonstrated this modulation so far.

Ataxin-2 also associates directly with DEAD/H-box RNA helicase DDX6 (*Nonhoff et al., 2007; Kaehler et al., 2012*), which intervenes in several stages of RNA processing. DDX6 is associated with mRNA decapping processes and translation repression through the miRNA pathway (*Coller et al., 2001; Chu and Rana, 2006; Mathys et al., 2014; Ayache et al., 201*5). DDX6 is part of ribonucleoprotein granules (P-bodies) promoting the sequestration of mRNAs and blocking its translation (*Coller et al., 2001; Fischer and Weis, 2002; Cougot et al., 2004; Andrei et al., 2005*). Moreover, DDX6 is also involved in mRNA stability, its increase in *C. elegans* leads to mRNA instability (*Hubstenberger et al., 2015*) and its silencing in mammalian cells can boost translation (*Chu and Rana, 2005*). Ataxin-2 mutation might impair the interaction with DDX6. In peripheral blood mononuclear cells of SCA2 patients, DDX6 expression is decreased, possibly due to RNA disturbances in the disease (*Nkiliza et al., 2016*). Moreover, ATXN2L also

associates with DDX6 (*Kaehler et al., 2012*), ensuring the importance of this binding to the preserved role of ATXN2 in RNA metabolism.

Furthermore, ataxin-2 binds directly with RNA splicing factors with RNA recognition motifs (RRMs) such as RNA-binding motif protein 9 (RBM9), RNA binding protein with multiple splicing sites (RBPMS) (*Lim et al., 2006*), TAR DNA-binding protein 43 (TDP-43) (*Elden et al., 2010*) and RRM- containing fused in sarcoma protein (FUS) (*Farg et al., 2013*) (Figure 4). It is difficult to distinguish the exact effect of ataxin-2 on RNA processes coordinated by these proteins, however the regulatory effect of ataxin-2 over these RNA-binding proteins further supports the involvement of ataxin-2 in RNA metabolism.

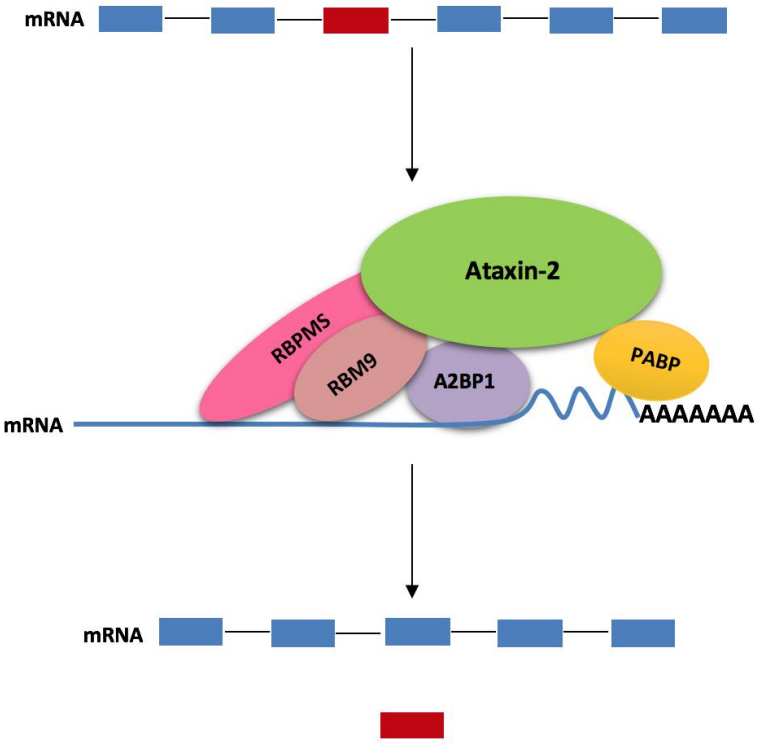

Figure 4. Ataxin-2 mediates RNA metabolism processes. Interaction of ataxin-2 with proteins involved in mRNA alternative splicing implies a role for ataxin-2 in posttranscriptional modifications. Ataxin-2 is also able to directly bind to mRNA. Adapted from (Magaña et al., 2013).

1.1.5.3. Ataxin-2 regulates translation

Studies in yeast reported Pbp1 and Pab1 (PABP homologue) interaction as determinant for translation regulation. The poly(A) tail of a mRNA influences translation initiation and the rate of the poly(A) tail removal determines how fast a mRNA is degraded (*Jacobson., 1996; Xie, 2014*). In this paradigm, authors observed that Pab1 deletion can inhibit translation and the deletion of Pbp1 can suppress the lethality caused by Pab1 absence (*Mangus et al., 1998*).

Pbp1 was found to mimic the effect of cyclohexamide treatment, a translation inhibitor (*Dunn and Jensen, 2003*). Decreased Pbp1 function can alter the abundance of particular proteins without altering the abundance of the corresponding transcript, supporting a role as a translation regulator (*Ciosk et al., 2004*).

In 2006, a study by Satterfield and Pallanck showed that ataxin-2 (human) and dATX2 (*Drosophilla*) interact directly with translational machinery by assembling with polyribosomes, through the Lsm/LsmAD domain and the PAM2 motif independently (*Satterfield and Pallanck, 2006*). On the other hand, through the PAM2 domain, the authors propose that dATX2 is able to assemble to mRNA poly(A)-bound dPABP, promoting or preventing the interaction between dPABP and the translation initiation complex. Moreover, the interaction of dATX2 with the translational machinery through the Lsm/LsmAD domain might increase mRNA stability and/or mediate RNA-RNA interactions, facilitating microRNA targeting hence inhibiting translation (*Satterfield an Pallanck, 2006*). In line with this, ataxin-2 knockout impairs amino acid incorporation during mRNA translation, compromising protein synthesis (*Fittschen et al., 2015*) (Figure 5).

Ataxin-2 also interacts with fragil X mental retardation protein (*dFMR1*), DEAD box helicases of the Me31B family, GW182, argonaute 1 (*ago1*) and DDX6, implicated in neuronal translational control and miRNA function (*Ciosk et al., 2004; Nonhoff et al., 2007; Kozlov et al., 2010; McCann et al., 2011; Sudhakarn et al., 2014; Kamenska et al., 2016*). RNA interference (RNAi) pathway controls RNA stability and mRNA translation through miRNA (microRNA). The miRNAs, small noncoding RNAs, bind complementary sequences in mRNA 3' UTRs and repress translation via the RNA-induced silencing complex (RISC) (*Buchan, 2014*). In Drosophila, dAtx2 is necessary for miRNA function and synapse-specific long-term habituation (LTH), through miRNA-mediated translational repression (*McCann et al., 2011*). The authors of this study suggest that the miRNAs and RISC factors, such as Me31B, facilitate the recruitment of ataxin-2 to the mRNA 3'UTR where it interacts with PABP to suppress translation. This translation repression could happen through the disruption of PABP and eukaryotic initiation factor 4G (eIF4G) binding or through the recruitment of deadenylases that promote mRNA deadenylation and consequent repression (Figure 5) (*McCann et al., 2011*). Furthermore, in a MJD mouse model, the ataxin-2 levels are lower (*Nobrega et al., 2015*). This depletion promotes PABP increase, resulting in an increased translation of ataxin-3 (*Nobrega et al., 2015*). In this study, the reestablishment of ataxin-2 reduced ataxin-3 levels, an effect lost upon mutation of the PAM2 within ataxin-2. These results support that the ataxin-2-PABP interaction represses translation (*Nobrega et al., 2015*).

In eukaryotic cells, non-translating mRNAs can accumulate in mRNP granules: P-bodies and stress granules. Ataxin-2 can act as a negative repressor of translation since it is a constitutive part of stress granules (*see next topic for further details*). Stress granules contain mRNAs stalled in initiation translation, that when sequestered to these structures don't advance in this process (*Anderson and Kedersha, 2009a,b; Buchan and Parker, 2009; Protter and Parker, 2016*). Interestingly, several of ataxin-2 interacting factors can also be found in stress granules and/or promote the redirection of mRNAs to these structures, orchestrating translation arrest in a more or less ataxin-2 mediated manner (*Ralser et al., 2005a; Barbee et al., 2006; Nonhoff et al., 2007; Anderson and Kedersha, 2009a,b; Buchan and Parker, 2009; Protter and Parker, 2016*).

Altogether, these evidences suggest that ataxin-2 might be involved in translational regulation, through direct RNA binding and stabilization, and through several binding partners.

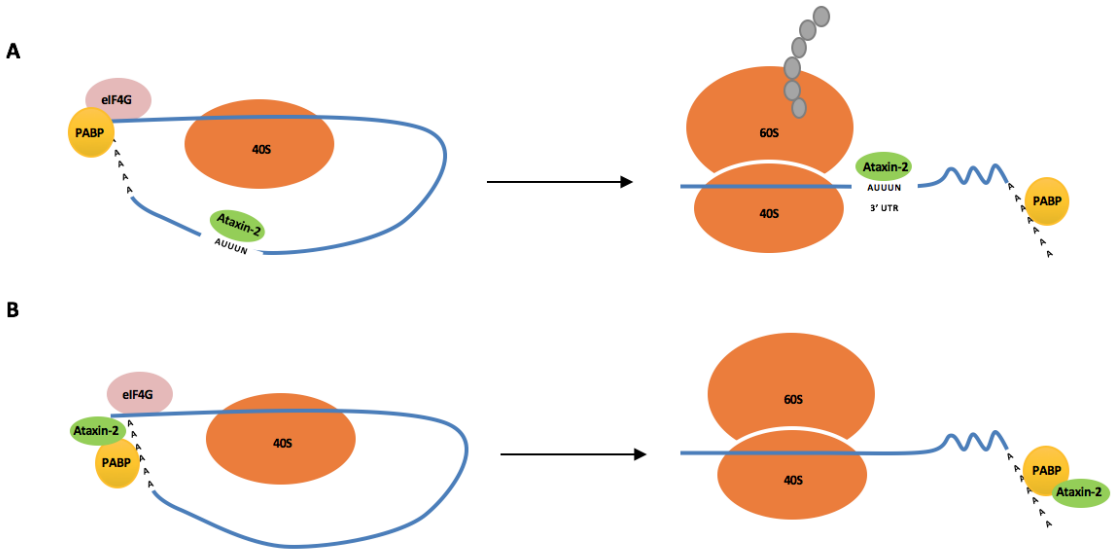

Figure 5. Ataxin-2 has a dual role in translation regulation. (A) Ataxin-2 promotes protein synthesis by directly binding to the 3'UTR of the mRNA. Ataxin-2 binding to mRNA increases stability and allows PABP to interact with translation initiation factor. This interaction promotes translation. (B) Ataxin-2 blocks protein synthesis through PABP binding. Ataxin-2 binds to mRNA-bound PABP trough the PAM2 motif and prevents the interaction between PABP and eIF4. Ataxin-2 binding to PABP in the translational apparatus blocks translation**.** eIF4G: Eukaryotic translation initiation factor 4 G; PABP: Poly(A) binding protein; 40S: Small ribosomal subunit; 60S: Large ribosomal subunit. Based on (McCann et al., 2011; Yokoshi et al., 2014)

1.1.5.4. Ataxin- 2 orchestrates stress granules formation

Ataxin-2 involvement in stress granules formation has been a hot topic of research, generating relevant evidences in different models (*Kedersha and Andersson, 2002; Ciosk et al., 2004; Ralser et al., 2005a; Lim et al., 2006; Kozlov et al., 2010; Swisher and Parker, 2010; Takahara and Maeda, 2012; Lastres-Becker et al., 2016*).

Stress granules formation is a cellular response mechanism towards stress, in which the cell conserves its energy by limiting the translation of non-necessary mRNAs, producing just the essential proteins for survival (*Lindquist, 1981; Li et al., 2013*). Stress granules form in response to a wide number of environmental stresses, such as heat shock, glucose deprivation, oxidative stress or drugs (*Anderson and Kedersha, 2009a,b; Protter and Parker, 2016*). Stress granules can be induced by pharmacological translation inhibition, by blockade of translation initiation factors or even by the overexpression of certain RNA-binding proteins (*Anderson and Kedersha, 2008; Li et al., 2013*) (Figure 6).

Stress granules are dynamic, containing a core structure with molecules such as PABPC1 or G3BP stress granule assembly factor 1 (G3BP1) and an outside pool of molecules that adjust and exchange with the surrounding cytosol (*Souquere et al., 2009; Buchan, 2014; Jain et al., 2016; Protter and Parker, 2016*). The content of stress granules is quite unique: stalled mRNAs not trapped in polysomes (*Parker and Sheth, 2007*), translation initiation factors, post-translation modification enzymes, metabolic enzymes, protein or RNA remodeling complexes and around 50% of its content includes RNA-binding proteins (*Anderson and Kedersha, 2009a,b; Jain et al., 2016; Protter and Parker, 2016*). Furthermore, it is also noteworthy the fact that stress granules content might vary upon different types of stress and cell type. Stress granules formation can therefore promote different outcomes in different organisms and upon different types of stress (*Buchan and Parker, 2009; Li et al., 2013; Protter and Parker, 2016*).

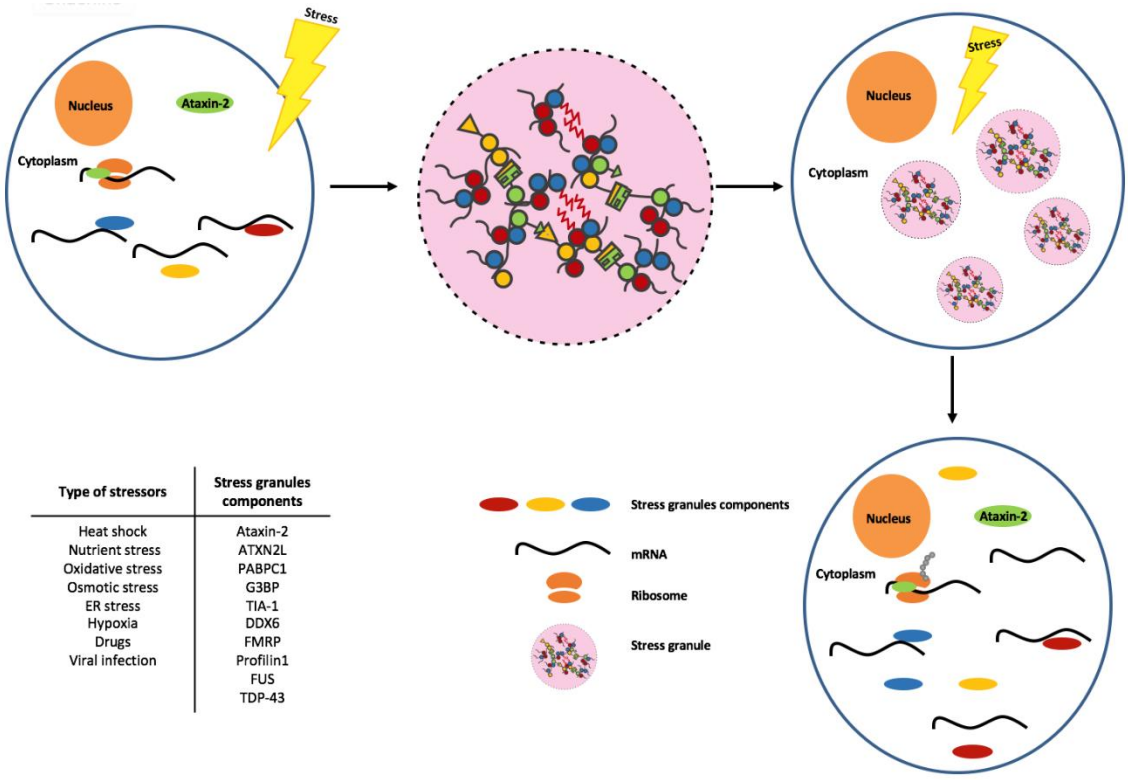

Figure 6. Stress granules assembly. Upon stress, the cellular response is to block protein synthesis in order to preserve energy for survival mechanisms. In order to stop protein synthesis, mRNAs, RNA-binding proteins, ribosomal units and translation initiator factors assemble to form cytoplasmatic granules named stress granules. Stress granules keep the mRNAs and factors necessary for protein synthesis stalled for as long as the stress endures. Once the stress is surpassed, stress granules disassemble and translation resumes. Stress granules are also a form of negative regulation of translation. Stress granules composition varies with the type of stress. Based on (Protter and Parker, 2016)

Stress granules assembly and disassembly is a complex and not completely understood process. Ataxin-2 and its orthologs have been extensively described has integrative part of these RNA-mediated stress response. Ataxin-2/Pbp1 overexpression can promote stress granules and its absence or depletion leads to a decrease in this formation, however ataxin-2 is not completely required for SGs assembly (*Ciosk et al., 2004; Nonhoff et al., 2007; Buchan et al., 2008; Grousl et al., 2009; Tharun et al., 2009*; *Swisher and Parker, 2010; Lastres-Becker et al., 2016*). Ataxin-2 interactors, such as PABPC1, DDX6, GW181, Me31B, TDP-43 and FUS protein, are also present in stress granules (Table 2) (*Ralser et al., 2005a; Nonhoff et al., 2007; Kozlov et al., 2010; Parker and Sheth, 2007; Li et al., 2013; Aulas and Vande Velde, 2015*). It is possible that the role of ataxin-2 in stress granules formation depends on those interactions, whether by RNA-binding or by protein-protein interactions.

Table 2. Ataxin-2 interactors as stress granules components

Protein	*Role*	*Related with stress granules in*	*Reference*
PABP	RNA-binding protein Translation initiator	Yeast, COS7, Hela and Du145 cell lines	*Kedersha et al., 2000; Kedersha et al., 2005; Swisher and Parker, 2010*
ATXN2L	Ataxin-2 paralog	HEK293T, HeLa and SH-SY5Y cell lines	*Kaehler et al., 2012*
DDX6	miRNA function	Du145, SH-SY5Y and HEK293T cell lines	*Nonhoff et al., 2007; Kaehler et al., 2012*
FUS	RNA-binding protein	Mouse, HeLa and SH-SY5Y cell lines, iPSCs from ALS patients	*Takanashi and Yamaguchi, 2014; Baron et al., 2013; Daigle et al., 2013; Lenzi et al., 2015*
TDP-43	DNA- and RNA-binding protein	Mouse, HeLa and HEK293T cell lines, ALS patients lymphoblasts	*Colombrita et al., 2009; Liu-Yesucevitz et al., 2010; McDonald et al., 2011; Aulas et al., 2012;*
FMRP	RNA-binding protein	Fly, mouse and human oocytes	*Mazroui et al., 2002; Gareau et al., 2013; Rosario et al., 2016*
Profilin1	Actin-binding protein	Yeast, HeLa and U2OS cell lines	*Figley et al., 2014*

1.1.5.5. Ataxin-2 modulates cytoskeleton reorganization

The less explored cellular function of ataxin-2 is its putative role in cytoskeleton reorganization. In yeast lacking fimbrin, a protein involved in actin filament organization, the expression of ataxin-2 is toxic (*Ralser et al., 2005b*). Pbp1, was found to associate with actin patches (*Michelot et al., 2010*), to interact with α-actinin 1 and α-actinin 2 (*Lim et al., 2006*) and associate with profilin 1 (*Figley et al., 2014*). Profilin 1 associates with dynein (cytoskeleton component) and has a well-characterized role in the modulation of actin filaments (*Birbach, 2008; Alkam et al., 2016*). Both profilin 1 and Pbp1 are components of stress granules, some studies point to a role of microtubule cytoskeleton in stress granules assembly/disassembly (*Ivanov et al., 2003; Kwon et al., 2007; Kolobova et al., 2009; Loschi et al., 2009; Rajgor and Shanahan, 2014*). Pbp1 may regulate profilin 1 in the assembly and bundling of actin filaments (*Figley et al., 2014*) and perhaps, both might be involved in cytoskeleton reorganization upon cellular stress.

Recent studies in *C. elegans* suggest that ataxin-2 is relevant for the microtubule dynamics during cell cycle (*Gnazzo et al., 2016; Stubenvoll et al., 2016*). Centrosomes orchestrate microtubules dynamics to establish bipolar spindles in mitosis. Errors in centrosome association lead to failure in the integration of genomic content and consequently, aneuploidy (*Nigg and Stearns, 2011*). Several RNA-binding proteins have been correlated with the control

of centrosome size and microtubule dynamics (*Filippova et al., 2012; Ishigaki et al., 2014*). At centrosomes, ATX-2 associates with γ Tubulin and was shown to interact with *szy-20* (controls directly centrosomal proteins), serving as a negative regulator of centrosome size (*Stubenvoll et al., 2016*). The authors propose that SZY-20 positively regulates ATX-2 to maintain centrosome size and microtubule dynamics (*Stubenvoll et al., 2016*) (Figure 7).

Furthermore, ataxin-2 had already been described as necessary for cytokinesis in mammalian cells (*Skop et al., 2004*). Cytokinesis is the physical process at the end of cell cycle, which divides the cytoplasm of a parental cell into two daughter cells. The process of cytokinesis is dynamic and requires the assembly of several proteins, namely actin filaments. A recent study in *C. elegans* further explored ATX-2 role in this process. ATX-2 assembles around mitotic structures such as the centrosome and the spindle (*Gnazzo et al., 2016*). The stability of these structures is determinant for a successful cell division (*Lewellyn et al., 2011; White and Glotzer, 2012; Landino and Ohi, 2016*). Moreover, ATX-2 facilitates cytokinesis and promotes cell division by the targeting ZEN-4 to the spindle midzone through the post-transcriptional regulation of PAR-5 (*Gnazzo et al., 2016*) (Figure 7).

Furthermore, in a *Drosophila* study, *dAtx2* was suggested as a dosage-sensitive regulator of actin filaments. Dosage alterations in *dAtx2* lead to modifications in actin filaments, causing apoptosis in retinal cells and sterility. The authors purpose dAtx2 involvement not in production of actin but rather in its organization, by translational regulation of actin polymerization mediators (*Satterfield et al., 2002*). dAtx2 also enhanced taupathology (*Shulman and Feany, 2003*), alterations in Tau can reduce its microtubule affinity and interaction, promoting oligomerization and toxicity (*Hasegawa et at., 1998*). dAtx2 might interfere with Tau's ability of interacting with microtubule, being responsible for the toxicity.

In mammalian cells (cell line and mouse brain), ataxin-2 interacts with plastin (*Ralser et al., 2005b*), protein involved in cytoskeleton organization and stabilization. Plastin binds directly to actin promoting stability and bundling (*Shinomiya, 2012*), ataxin-2 might mediate this interaction.

Altogether these evidences point to an involvement of ataxin-2 in cytoskeleton reorganization, acting probably as a modulator of microtubule structures by translational regulation or through protein-protein interactions.

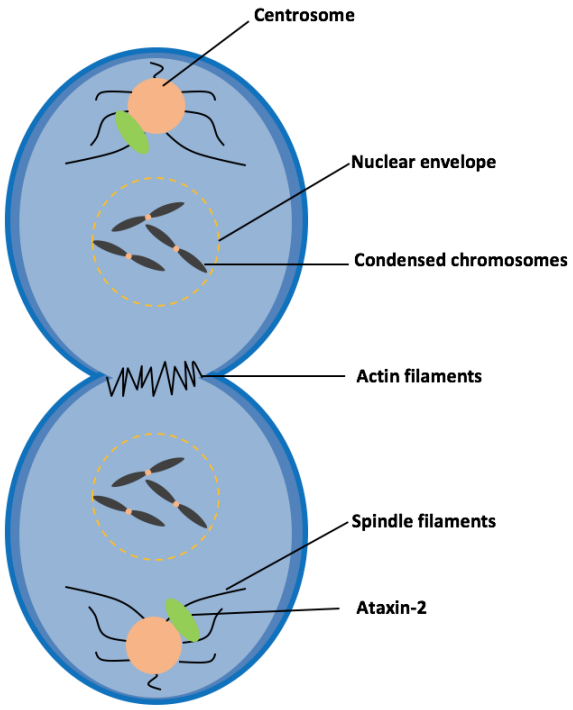

Figure 7. Ataxin-2 mediates cellular division. Ataxin-2 interaction with microtubules strongly supports a role for cytoskeleton organization. In *C. elegans*, ataxin-2 can associate with the centrosome and spindle (composed by microtubules), regulating the size and stability of these structures and ensuring cellular division. Ataxin-2 also participates in the process of cytokinesis, that involves the reorganization of the actin filaments to allow the physical separation of the cells. Based on (Gnazzo et al., 2016; Stubenvoll et al., 2016)

1.1.5.6. Ataxin-2 participates in endocytosis

Endocytosis is an active mechanism through which cells engulf and internalize molecules, macromolecules, particles and fluids in vesicles formed by a portion of plasma membrane. Then, the vesicles content is sorted and either delivered to the lysosome for degradation or recycled back to the plasma membrane. At the signalling level, endocytosis removes activated ligand-receptor complexes from the cell surface allowing cells to receive additional signals. It is still controversial the purpose of this process; whether it serves to neutralize receptor binding or to improve receptor-ligand signalling in a fast and effective manner (*Wiley and Burke., 2001*).

Ataxin-2 is known to interact with endophilins A1 and A3 through the SH3 domains (Src Homology 3) (*Ralser et al., 2005b; Lim et al., 2006; Nonis et al., 2008*). Endophilins are proteins implicated in the formation of the plasmatic membrane curvature in the endocytosis process and endophilin A family is also connected to clathrin-mediated endocytosis (*Ringstad et al., 1999; Fabian-Fine et al., 2003; Kjaerulff et al., 2011*).

A study suggested that ataxin-2 association with endophilin A influences membrane trafficking and postsynaptic membrane receptor density, modulating neuronal signalling (*Nonis et al., 2008*). Considering that epidermal growth factor receptor (EGFR) internalization by endocytosis is well known, these authors used this model to study ataxin-2 role in endocytic machinery. Ataxin-2 overexpression decreased EGFR internalization at plasma membrane, whereas ataxin-2 absence enhanced EGFR internalization (*Nonis et al., 2008*). The authors proposed that the domains of ataxin-2, SH3 and SH2 (Src Homology 2), can bind with activated/phosphorilated receptor tyrosine kinases (RTKs) such as the EGFR (*Nonis et al., 2008*). SH3 domains bind proline-rich regions and SH2 domains are related with tyrosine-rich domains binding (*Wiley and Burke., 2001*). Several authors propose that RTKs endocytosis might share the same effectors (*Wiley and Burke., 2001*); which means that ataxin-2 ability to bind to SH3 domains implicates it specifically in RTKs signalling (Figure 8).

Upon growth factors binding to plasma membrane receptors, endophilins A associate with adaptor proteins (Scr, Grb2) to internalize the ligand-receptor complex. Drost and colleagues described the decrease of protein adaptors Grb2 and Scr in the brain of ATXN2 KO mice and in fibroblasts of SCA2 patients (*Drost et al., 2013*). The fact that these two proteins are specifically affected supports ataxin-2 as determinant for endocytosis.

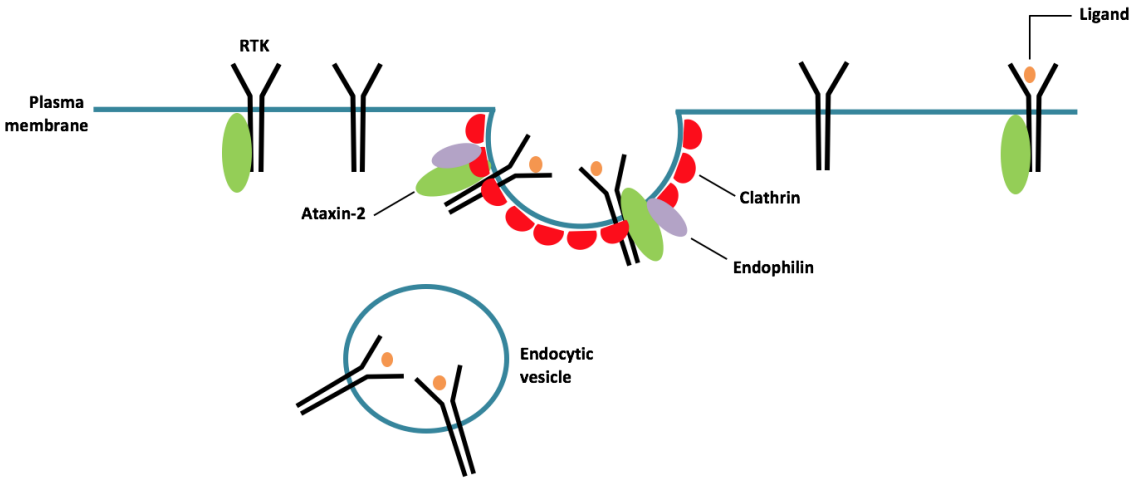

Figure 8. Ataxin-2 mediates RTK endocytosis. Ataxin-2 binds tyrosine kinase receptors (RTK) trough the SH3 and SH2 domains modulating this receptor-mediated signalling. Ataxin-2 associates with elements such as endophilins to allow the internalization of the ligand-receptor structure. Based on (Nonis et al., 2008; Drost et al., 2013)

1.1.5.7. Ataxin-2 mediates calcium signalling

Calcium signalling has long been described as deranged in polyglutamine disorders such as HD, MJD and also SCA2 (*Bezprozvanny and Hayden, 2004; Chen et al., 2008; Liu et al., 2009;*

Bezprozvanny, 2011; Kasumu et al., 2012a; Kasumu et al., 2012b; Kasumu and Bezprovanny; Swarup et al., 2013; Halbach et al., 2016). In SCA2, the high impact that the mutation of ataxin-2 has on calcium homeostasis might indicate a critical role for this protein in calcium signalling (Figure 9).

Calcium signalling involves several channels such as: the voltage-dependent calcium channels (VDCC), NMDA receptors, AMPA receptors, transient receptor potential channels (TRP), small conductance calcium channels (SK). The Inositol 1,4,5-Trisphosphate Receptor Type 1 (ITPR1) of the endoplasmic reticulum controls the release of calcium (reviewed in *Bezprozvanny, 2010*).

In *SCA2–58Q* transgenic mouse model, authors describe a supranormal cytosolic concentration of calcium and report that mutant ataxin-2 interacts with the carboxyl-terminal region of the ITPR1 of the calcium channel in the endoplasmic reticulum (*Liu et al., 2009; Kasumu et al., 2012a*). Moreover, mutant ataxin-2 seems to display higher binding affinity to ITPR1, which suggests that this binding could be mediated by the polyQ expanded tract (*Kasumu et al., 2012a*). Factors involved in calcium homeostasis, such as ITPR1, ATPase Sarcoplasmic/Endoplasmic Reticulum Ca^{2+} Transporting 2 (Atp2a2), Inositol Polyphosphate-5-Phosphatase A (Inpp5a) and RAR Related Orphan Receptor A (Rora), were recently found to be downregulated both in the cerebellum of ATXN2 KO mice and in GAG42-knock-in mouse model of SCA2 (*Halbach et al., 2016*). Moreover, modulation and stabilization of calcium channels, the inhibition of ITPR1 (*Kasumu et al., 2012a*) and the activation of SK (*Kasumu et al., 201b*), alleviated motor deficits in SCA2–58Q mice.

In humans, a polymorphism in the calcium voltage-gated channel subunit alpha 1A (CACNA1A) is correlated with the age of onset of SCA2 patients. The increased number of CAG repeats in this gene can be directly correlated with the earlier onset of SCA2 (*Pulst et al., 2005*). A study in blood of SCA2 patients, also described an alteration in the subunit γ3 of the VDCC, supporting a major role of ataxin-2 in calcium signalling (*Swarup et al., 2013*).

Purkinje cells in the cerebellum are the main affected cells in SCA2. These cells have high ITPR1 levels (*Furuichi et al., 1989; Mignery et al., 1989*) so are particularly sensitive to calcium signalling. Therefore, it is possible that the marked neurodegeneration observed in SCA2 results from impaired calcium signalling, with an increase of glutamate leading to excitotoxicity and apoptosis. Although there are no direct reports regarding wild type ataxin-2 and calcium signalling, the impairment on calcium homeostasis caused by a mutation in this protein provides strong evidence of ataxin-2 determinant role in these processes.

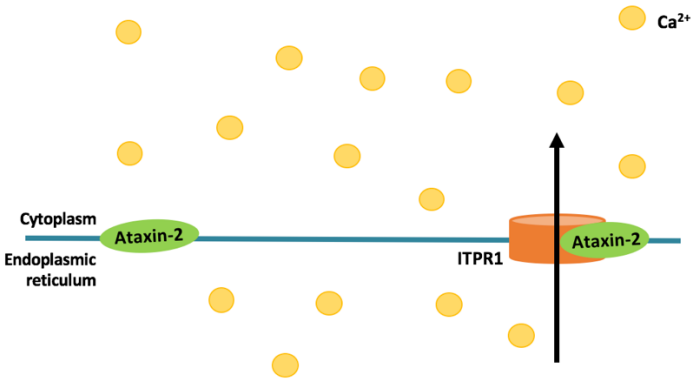

Figure 9. Ataxin-2 mediates calcium signalling. Studies with mutant ataxin-2 suggest that ataxin-2 is able to modulates the release of calcium from the endoplasmic reticulum through the binding to the Inositol 1,4,5-Trisphosphate Receptor Type 1 (ITPR1). Adapted from (Magaña et al., 2013)

1.1.5.8. Other functions

Ataxin-2, ataxin-2 orthologs/homologues/paralogs and its major interacting partners have been correlated with other cellular processes over the years. Since these reports usually refer to mutant ataxin-2 and are single reports, it is difficult to ascertain if these are conserved physiologic functions of this protein (Table 3).

Table 3. Reports relative to ataxin-2 impact on several cellular functions

Model	*Mechanism*	*Phenotype*	*Reference*
Drosophila	Translational regulation of synaptic mRNAs	Long-term olfactory habituation (LTH)	*McCann et al., 2011; Sudhakarn et al., 2014*
Mouse	Modulation of TKR endocytosis	Cellular growth, proliferation and differentiation	*Drost et al., 2013*
Humans (SCA2 patients)	Alteration in TGF-β1, ERK5 and HIF1α pathways	Cellular growth, proliferation and differentiation	*Nkiliza et al., 2016*
Drosophila	A2BP1 binding	Cellular proliferation	*Tastan et al., 2010; Hamada et al., 2013; Hamada et al., 2015*
Drosophila Humans (SCA2 patients)	E-cadherin interaction Alteration in cell adhesion pathways	Cellular adhesion	*Toret et al., 2014; Nkiliza et al., 2016*
Humans	unknown	Apoptosis	*Wiedemeyer et al., 2003*
Yeast Mouse Humans	Fat distribution, Lipid metabolism, insulin signalling, mTOR regulation	Metabolism regulation	*Kiehl et al., 2006; Lastres-Becker et al., 2008b; Damrath et al., 2012; Scoles et al., 2012; Fittschen et al., 2015; Meierhofer et al., 2016; Lastres-Becker et al., 2016; Carmo-Silva et al., 2017*

1.2. Ataxin-2 as a metabolic mediator

The generation of a mouse knockout for ataxin-2 (ATXN2 KO), by two different groups (*Kiehl et al., 2006; Lastres-Becker et al., 2008b*), resulted in interesting observations relative to ataxin-2 effects on the organism, mainly in body weight, insulin sensitivity and fertility. The alterations in body weight and metabolism were supported by subsequent studies in several animal models and observations in SCA2 patients (reviewed in *Carmo-Silva et al., 2017*).

In this section we present and discuss a possible contribution of ataxin-2 to the whole body homeostasis.

1.2.1. Role of ataxin-2 on body weight and adipose tissue distribution

A recent study in *C. elegans* showed that *atx-2*, the homolog of mammalian *ATXN2* gene, regulates animal size, fat content and rate of development (*Bar et al., 2016*). In this study, authors submited the animals to dietary restriction (DR), a well-known metabolic intervention that extends lifespan and decreases body size and fat accumulation. Down-regulation of *atx-2* led to fat accumulation and growth increase even in a DR regimen. On the opposite, overexpression of *atx-2 per se* led to a decrease in body size and fat accumulation, mimicking DR effects. These effects might be correlated with the mTOR (mechanistic target of rapamycin) pathway, as a key regulator of growth and metabolism. Atx-2 overexpression can inhibit mTOR signalling, probable being the cause for the impact over body weight and fat accumulation. In fact, authors suggest that ataxin-2 might be a mediator of DR beneficial effects (*Bar et al., 2016*).

The Atxn2-CAG42-Knock-In mice showed a significant lower body weight when compared to wild type littermates as early as the age of 10 days (*Damrath et al., 2012*). A more recent study, using a transgenic BAC-SCA2 mouse model reported that mutant ataxin-2 expression resulted in lower body weight when compared with non-expanded ataxin-2 expression (*Dansithong et al., 2015*). These observations suggest that abnormal ataxin-2 function or even loss of function change metabolic homeostasis leading to weight alterations.

ATXN2 KO mice develop obesity (*Kiehl et al., 2006*, *Lastres-Becker et al., 2008b*). Kiehl and colleagues suggested that increased body weight might be caused by hyperphagia (*Kiehl et al., 2006*). On the opposite, Lastres-Becker and colleagues did not report hyperphagic behaviour in these animals, but observed that the mice were hyperactive raising some controversy about the cause of obesity (*Lastres-Becker et al., 2008b*). In 2012, Scoles and colleagues reported

hyperphagia in ATXN2 KO and even heterozygous mice (ATXN2$^{+/-}$) (*Scoles et al., 2012*). These authors suggest that the expression of ataxin-2 in cells in the nose and mitral cells of the olfactory bulb might explain obesity; since the loss of ataxin-2 might compromise olfaction and diminish reward signals mediated by smell, causing the hyperphagia (*Scoles et al., 2012*). In fact, others showed in *Drosophila* that ataxin-2 is necessary for olfactory habituation (*McCann et al., 2011; Sudhakarn et al., 2014*) and olfactory impairments were described in SCA2 patients (*Velazquez-Perez et al., 2006*).

Several known direct mediators of ataxin-2 functions had already been implicated in models of anorexia and obesity. The analysis of transcriptome of an anorexia mouse model, *anx/anx* mice, found upregulation of *Dddx6* and *Pabpc1* (*Mercader et al., 2012*), both shown to interact directly with ataxin-2 and to be affected by its modulation (*Albrecht et al., 2004b; Satterfield and Pallanck, 2006; Nonhoff et al., 2007*). A polymorphism in *A2BP1* gene, a known associated of ataxin-2 (*Shibata et al., 2000*), is associated with obesity (*Ma et al., 2010*). Furthermore, a different polymorphism in A2BP1 was also correlated with antiphycotic-induced weight gain (*Dong et al., 2015*). However, A2BP1 knockdown in N-41 hypothalamic cell line also promotes the concomitant decrease of ataxin-2 (*Ma et al., 2010*), which might hint that upon A2BP1 loss of function a decrease in ataxin-2 occurs, thus contributing to the weight gain. Ataxin-2 overexpression can also increase TDP-43 cytoplasmic levels and prevent its assembly in RNP granules (*Nihei et al., 2012*). Interestingly, TDP-43 regulates glucose uptake in skeletal muscle, insulin sensitivity and fat distribution (*Stallings et al., 2013*).

Regarding body weight and food intake in polyglutamine disorders, the major observation is that patients have lower body weight and adipose tissue loss (*Auburger, 2012*). However, the causes and mechanisms underlying this body weight loss are not completely known, but could be related to the decreased ability to swallow food, that occurs in a later onset of these motor disorders, or the putative dysfunction of central appetite regulation system and energy homeostasis *per se*. In animal models, studies reported a loss of body weight, being the model of Huntington's the most studied case (*Van Raamsdonk et al., 2006*). Huntington's and SCA2 patients have increased appetite but a decrease in body weight and subcutaneous adipose tissue (*Trejo et al., 2004; Aziz et al., 2008*; *Auburger, 2012*). Interestingly, in 2008, Abdel Aleem and Zaki described a family of SCA2 patients that presented significant increase of appetite, polyphagia and consequent obesity. This was the first study to suggest an interaction between ataxin-2 and appetite-regulating genes (*Abdel-Aleem and Zaki, 2008*). To date, there are no further studies in SCA2 patients describing food intake and/or body weight changes.

Even though obesity is a multifactorial condition, it is known that there are some genes that contributes to the development of obesity (*Lu and Loos, 2013; Albuquerque et al., 2015*). Major gene mutations, a single nucleotide polymorphisms (SNPs) or modifications in several genes might determine a predisposition for obesity, body adipose tissue accumulation and distribution (*Albuquerque et al., 2015*).

Chromosome 12q contains one of the largest blocks of linkage disequilibrium (LD) in the human genome (*Scherer et al., 2006*). Several genetic linkage studies of human obesity-related traits have already implicated the chromosome region 12q23-24 with this obesity related phenotypes (*Chagnon et al., 2004; Li et al., 2004; Dong et al., 2005; Wilson et al., 2006*). Moreover, several studies correlated *ATXN2* or the *ATXN2/SH2B3* trait (*Auburger et al., 2014*) with increased risk to type 1 diabetes, obesity and its related disorders such as hypertension and coronary disease (Table 4).

Table 4. Metabolic alterations and metabolic-related disorders resulting from mutation and single nucleotide polymorphisms (SNP) in ATXN2 gene

Gene alteration		***Metabolic alterations and metabolic-related disorders***	***Reference***
***ATXN2* mutation in exon1** *Spinocerebellar ataxia type 2*		Weight loss	*Auburger, 2012*
		Polyphagia and Obesity Xanthomas	*Abdel-Aleem and Zaki, 2008*
		Increased malondialdehyde (MDA) (lipid peroxidation)	*Forment et al., 2010*
		Decreased Apolipoprotein C-II & C-III	*Swarup et al., 2013*
		Decreased Grb2 (tyrosine kinase receptors signalling)	*Drost et al., 2013*
Single nucleotide polymorphisms *ATXN2*	rs11065979	Obesity Increased blood pressure	*Chagnon et al., 2004* *Franceschini et al., 2016*
	rs10774625	Increased blood pressure Cardiovascular disease	*Ikram et al., 2010*
	rs695872	Obesity in children	*Figueroa et al., 2009*
	rs653178	Increased blood pressure Cardiovascular disease Myocardial infarction Metabolic syndrome	*Ganesh et al., 2013* *Zhang et al., 2015* *Gudbjortsson et al., 2009* *Kraja et al., 2014*
	rs7137828	Glaucoma	*Cooke Bailey et al., 2016*

These studies in mice and humans suggest a role for ataxin-2 as regulator of body weight. Even though the mechanisms underlying the pathology of SCA2 are not yet completely understood, it is possible that the extent of polyQ tract differs between individuals leading to different

effects on body weight. Moreover, the regulation of body weight and food intake comprises a complex network of central and peripheral signals, affected by genetics, behavioural and environmental conditions. The role of ataxin-2 on a possible regulation of neuropeptides or hormones involved in food intake remains to be investigated.

1.2.2. Ataxin-2 modulates lipid metabolism

The ATXN2 KO mouse phenotype suggests a role for ataxin-2 on lipid metabolism (*Lastres-Becker et al., 2008; Meierhofer et al., 2016*). Lastres-Becker and colleagues (2008) observed that ATXN2 KO mouse presented lipids and glycogen accumulation in the liver and consequent hepatosteatosis. Moreover, these mice presented and hepatic increase in insulin-induced gene 1 (Insig1), which could be a consequence of increased circulating insulin (*Lastres-Becker et al., 2008b*).

Insig1 is regulated by insulin levels and is highly expressed in the liver, being responsible for the negative regulation of adipocyte differentiation, lipogenesis, cholesterol synthesis and triglycerides processing (*Yamashita et al., 2003*). Some authors suggest that an increase on Insig1 levels is a physiological response to fight obesity and restore insulin signalling (*Li et al., 2003*). Moreover, since ataxin-2 is involved in the endocytosis of RTKs, the process of insulin receptor (InsR) endocytosis might be compromised by the absence of ataxin-2 (*Lastres-Becker et al., 2008b*). The impairment of InsR endocytosis compromises insulin signalling and induces insulin resistance (as explored in *1.2.3*). Insulin resistance is one of the major mechanisms underlying lipid metabolism dysfunction and hepatosteatosis. In more detail, decreased insulin function induces: (i) decreased triglycerides storage in adipose tissue, increasing triglycerides circulating levels; (ii) decreased glucose uptake in different cells; (iii) lipolysis increase; (iv) decreased glycogen synthesis (*Samuel and Shulman, 2012; Chavez and Summers, 2012; Sun and Lazar, 2014*).

ATXN2 KO mice have higher levels of serum cholesterol and changes in lipids content in the cerebellum (ceramide, sphingomyelin, gangliosides, sulfatides, cholesterol, sphingomyelinases and PPARδ), which might impair neuronal signalling at membrane levels (*Lastres-Becker et al., 2008b*). This study reported, for the first time, the involvement of ataxin-2 on body weight regulation as consequence of lipid metabolism changes.

More recently, proteomic and metabolomic studies in the ATXN2 KO mice supported this possible role of ataxin-2 in lipid metabolism (*Meierhofer et al., 2016*). The authors describe a strong downregulation in branched chain amino acid (BCAA) pathways, fatty acid metabolism,

and citric acid cycle in the liver. Moreover, observed a downregulation of ACADS (Acyl-CoA dehydrogenase, C-2 to C-3 short chain), which catalyses the fatty acid beta-oxidation, as responsible for the accumulation of fat droplets in the liver of these mice (*Meierhofer et al., 2016*). An increase in BCAA levels in blood is associated with obesity and metabolic stress (*Lynch and Adams, 2014*), on the contrary, depletion of BCAA levels levels are associated with body weight loss in Hungtington's disease patients (*Mochel et al., 2007; Mochel et al., 2011*). These apparently contradictory results are explained by the authors as a possible result of ataxin-2 homologues action that might rescue ataxin-2 function, or through alterations in mTOR signalling upon absence of ataxin-2 (as discussed in *1.2.4*) (*Meierhofer et al., 2016*).

Recent evidences further support ataxin-2 as a player in lipid metabolism (*Fittschen et al., 2015; Halbach et al., 2016*). Ataxin-2 depletion modifies the abundance of the lipid droplet regulator, perilipin-3 (*Plin3*), and the apolipoprotein secretor factor *Mttp* (Microsomal triglyceride transfer protein), suggesting that ataxin-2 participates in lipid energy reserves recruitment, possibly through a mRNA regulation process (*Fittschen et al., 2015*). Accordingly, ATXN2 KO mice have lower levels of *RORA*, a gene related to calcium signalling, lipid metabolism and circadian rhythm (*Halbach et al., 2016*). Remains to be understood if there is a direct connection between ataxin-2 and RORA in lipid metabolism and if so, how it is modulated.

As previously mentioned, progressive body weight changes and lipid pathology are also observed in SCA2 patients (*Abdel-Aleem and Zaki, 2008; Auburger, 2012*). Moreover, Abdel-Aleem and Zaki observed the presence of xanthomas on bony prominence in an Egyptian family with SCA2 that are also polyphagic (*Abdel-Aleem and Zaki, 2008*). Xanthomas are depositions of lipid-containing foam cells localized mainly in joints and tendons, generally associated to hyperlipidaemias and lipid metabolism disturbances (*Kedar and Gardner, 2013*). Moreover, SCA2 patient blood present increase in malondialdehyde (MDA) (*Forment et al., 2010*), and altered levels of apolipoproteins involved in lipid storage and trafficking (*Swarup et al., 2013*). In this study, the authors also observed a decrease in apolipoprotein A-1 (APO A-1) which is a component of high-density lipoprotein (HDL). A decrease in APO A-1 is also related to increased risk for cardiovascular diseases (*Ng et al., 1996*; *Mangaraj et al., 2016; Sorci-Thomas and Thomas, 2016*). The same study also described a decrease in apolipoproteins C-II and C-III (*Swarup et al., 2013*), responsible for maintaining triglycerides and free fatty acids levels in plasma. Interestingly, alterations in these proteins are usually associated to dyslipidaemia (*Mendivil et al., 2010*).

Altogether, the effect of ataxin-2 absence or mutation on lipid metabolism, suggests a relevant role of ataxin-2 on lipid storage and trafficking

1.2.3. Ataxin-2 as a mediator of insulin signalling

Insulin resistance is one of the major pathophysiologic conditions observed in metabolic syndrome and responsible for the worsening of the metabolic condition, promoting more dysregulation. Obesity is one of the main causes for insulin resistance and for type 2 diabetes (*Kahn et al., 2006*). Insulin resistance has been linked to different clinical conditions such as hypertension, dyslipidaemia, cardiovascular diseases, infertility and cancer (*Brüning et al., 2000*; *Kaaks et al., 2000; Mokdad et al., 2003; Donadon et al., 2009; Yoon et al., 2015; Hirabayashi, 2016; Janus et al., 2016; Meikle and Summers, 2016*).

To better understand the mechanism through which the absence of ataxin-2 causes a decrease in insulin sensitivity, it is relevant to highlight the mechanisms that promote insulin resistance: (i) damage/mutations in IR or IR substrates (IRS), (ii) downregulation of IR expression, (iii) defects in kinase activity or in intracellular signalling, (iv) negative regulation by hyperglycaemia, (v) lipotoxicity, (vi) inflammation, (vii) mitochondrial dysfunction and reactive oxygen species (ROS) formation and (viii) ER stress (*Boucher et al., 2014*).

Lastres-Becker and co-workers observed that ATXN2 KO mice were insulin resistant (*Lastres-Becker et al., 2008b*). In this study, the authors reported that ATXN2 KO mice have higher levels of blood insulin and pancreatic β cells enlargement, suggesting an increase in insulin production. They also observed a decrease in protein levels of InsR in the brain and liver, despite the increase in *InsR* mRNA, what could explain the insulin resistance of ATXN2 KO mice (*Lastres-Becker et al., 2008b*).

Ataxin-2 absence causes a decrease in InsR protein levels without altering mRNA levels, which suggests a post-transcriptional effect, or the lack of it (*Lastres-Becker et al., 2008b*). Ataxin-2 role in post-transcriptional modifications and translation regulation was already described (see *1.1.5* for detail). Moreover, it has been recently showed the interaction between ataxin-2 and the nutrient sensing pathway of mTOR (explored in *1.2.4*). The absence of ataxin-2 observed in ATXN2 KO mice induces hyperactivation of elements of the mTOR signalling pathway (*Lastres-Becker et al., 2016*). The abnormal activation of mTOR pathway can inhibit insulin signalling (*Um et al., 2004*). Briefly, in a normal condition, insulin binding stimulates PI3K that promotes Akt phosphorylation and activation, resulting in consequent mTOR activation. By feedback loop, mTOR and S6K1 will phosphorylate IRS1 at a serine residue that promotes insulin

desensitization. The abnormal overactivation of mTOR can therefore block the insulin signalling pathway and promote resistance (*Yoon and Choi, 2016*).

Upon ligand binding, InsR is internalized from the cell surface, similarly to other RTKs (*Morcavallo et al., 2014*). Defects in endocytosis of InsR might lead to deficient insulin signalling and insulin resistance (*Morcavallo et al., 2014; Boucher et al., 2014*). The process of InsR endocytosis is still not completely understood, the effectors of this process are still unknown. Ataxin-2 modulates the endocytosis of EGFR, an RTK (*Nonis et al., 2009*) (see *1.1.5.5*). Grb2 is an adaptor molecule involved in RTKs signalling that is decreased in fibroblasts of SCA2 patients (*Drost et al., 2013*). It is possible that by translation regulation, ataxin-2 controls Grb2 levels and interacts with this protein to orchestrate the InsR endocytosis. These observations might explain the insulin resistance observed upon ataxin-2 depletion/absence (*Lastres-Becker et al., 2008b*).

Inflammation, ER stress, mitochondrial dysfunction and ROS formation greatly contribute to insulin resistance (*reviewed in Boucher et al., 2014*). There are no current direct evidences linking ataxin-2 to these mechanisms, however, these are known to be relevant on pathophysiology of obesity (*Evans et al., 2005; Fridlyand and Philipson, 2006; Hotamisligil, 2010; Osborn and Olefsky, 2012*) and are also hallmarks of neurodegenerative mechanisms in polyglutamine disorders (*Orr and Zoghbi., 2007; Almaguer-Gotay et al., 2014*).

Dyslipidaemia induces and promotes insulin resistance (*Boucher et al., 2014; Meikle et al., 2014; Meikle and Summers, 2016*). As described in the previous section, ATXN2 KO mice present increased cerebellar lipids, namely sphingolipids such as ceramide, sphingomyelin and gangliosides (*Lastres-Becker et al., 2008b*). Increased sphingolipids are tightly correlated with obesity and insulin resistance (*Holland et al., 2007; Boucher et al., 2014; Meikle and Summers, 2016*). Ceramide increase impairs Akt activation and activates PKC (Protein kinase C) and JNK (c-Jun N-terminal kinase), promoting insulin resistance (*Schenk et al., 2008; Blouin et al., 2010; reviewed Boucher et al., 2014*).

Pharmacological or genetic approaches targeting enzymes required for the sphingolipid metabolism are known to improve insulin sensitivity (*Meikle and Summers, 2016*). Weight loss in obese individuals is enough to decrease serum levels of sphingomyelin and consequent insulin sensitivity increase (*Martinez-Ramirez et al., 2016*). Mice lacking GM3 ganglioside (monosialodihexosylganglioside), an enzyme necessary for the processing of ganglioside, maintain glucose tolerance and insulin response when challenged with high-fat diet

(*Yamashita et al., 2003*). The lipid accumulation observed in the brain of ATXN2 KO mice may promote insulin resistance by lipotoxicity (*Boucher et al., 2014; Meikle and Summers, 2016*).

Since insulin signalling is a complex and well-controlled mechanism, in ATXN2 KO mice it is not clear whether insulin resistance results from the obesity or if insulin resistance is at the base of obesity and metabolic dysfunction. It has been previously shown that neuronal insulin resistance may lead to obesity, hyperinsulinemia and dyslipidaemia as well as contribute to reproductive abnormalities (*Brüning et al., 2000; Biddinger and Kahn, 2006; Meikle and Summers, 2016*), in agreement with the findings in ATXN2 KO (*Lastres-Becker et al., 2008b*).

1.2.4. Ataxin-2 regulates metabolic pathways – stress granules as metabolic intermediates?

The most common and well-studied pathway regarding nutrient sensing is the mTOR (mechanistic target of rapamycin) signalling cascade. The mTOR pathway integrates signals downstream of insulin and other growth factors that reflect energetic availability (*Hu et al., 2016; Kennedy and Lamming, 2016*). In nutrient-rich conditions, the activation of mTOR pathway stimulates anabolic processes, such as protein and lipid synthesis in order to promote cell growth; whereas in nutrient-deficient conditions, anabolic processes are limited and catabolic processes such as autophagy, are activated to produce energy and nutrients for cell survival (*Malik et al., 2013*; *Shimobayashi and Hall., 2014; Switon et al., 2016*). The mTOR pathway is assured by the presence of two complexes, mTOR complex 1 (TORC1), and mTOR complex 2 (TORC2) (*Malik et al., 2013*; *Shimobayashi and Hall., 2014*). TORC1 is sensitive to rapamycin, and it is responsible for actions such as growth, translation regulation, ribosome biogenesis, nutrient transport and autophagy; whereas TORC2 is not sensitive to rapamycin and is mostly responsible for the cell cycle-dependent polarization of the actin cytoskeleton (reviewed in *Laplante and Sabatini, 2012; Kim et al., 2013; Porta et al., 2014*). Much has been studied regarding mTOR pathway regulation in stress, under normal cellular stress conditions and in disease (*Heberle et al., 2015; Wang et al., 2016*).

In the context of stress response, the first evidences connecting ataxin-2 with mTOR pathway were documented connecting it to stress granules formation (*Takahara and Maeda, 2012*). As mentioned before (see *1.1.5.4*), stress granules assemble upon stress as a cellular coping mechanism. One of the major roles of stress granules formation is translation blockade, so the cell will produce just the necessary proteins to survive the insult (*Kedersha and Anderson, 2007; Anderson and Kedersha, 2008; Buchan and Parker, 2009*). Stress granules are formed

under several stresses such as hypoxia, ER, oxidative, heat, cold, osmotic and nutrient stress (*Kedersha and Anderson, 2007; Anderson and Kedersha, 2008; Buchan and Parker, 2009; Protter and Parker, 2016*). It was also described that stress granules are able to sequester several signalling molecules, such as pro-apoptotic mediators (*Arimoto et al., 2008*). The components of stress granules depend on the type of stress (*Buchan and Parker, 2009; Buchan et al., 2012; Protter and Parker, 2016*).

Pbp1, the yeast ortholog of ataxin-2, has been described as a component of stress granules (*Buchan et al., 2008; Swisher and Parker, 2010*). In 2012 it was reported that Pbp1 might act as a negative regulator of TORC1 signalling by promoting its sequestration into stress granules (*Takahara and Maeda, 2012*). Several evidences support that Pbp1 controls TORC1 signalling by stress granules formation: (i) Pbp1 is able to induce stress granules in the absence of stress; (ii) Kog1 (ubiquitin-binding TORC1 subunit) and Tor1 (phosphatidylinositol kinase-related protein kinase) can be found in stress granules and co-immunoprecipitate with Pbp1; (iii) TORC1 is sequestered to stress granules upon heat stress; (iv) the disassembly of stress granules is sufficient for the recovery of TORC1 signalling (*Takahara and Maeda, 2012*).

A recent study showed that low energetic availability promotes the activation of the Snf1 pathway (AMPK ortholog in yeast), that leads to the phosphorylation of Pbp1 by Psk1 (PAS kinase ortholog). The phosphorylation of Pbp1 promotes TORC1 sequestration to stress granules, inhibiting mTOR pathway (*DeMille et al., 2015*).

The mTOR signalling pathway is one of the major nutrient-sensing pathways altered by dietary/caloric restriction (DR). A recent study in *C. elegans* suggested that ataxin-2 is an intermediate of dietary restriction-induced beneficial effects in metabolism (as discussed in *1.2.1*). Even though the authors do not investigate the effect dietary restriction on ataxin-2 expression (*Bar et al., 2016*), the DR-induced mTOR inhibition might be promoted by ataxin-2. Another recent work in mammalian cells further support this idea, showing that nutritional stress changes translation modulation through ataxin-2 (*Lastres-Becker et al., 2016*). The authors observed that in mouse embryonic fibroblasts (MEFs) from ATXN2 KO mice there is an hyperphosphorylation of ribosomal protein S6 (RPS6) and eukaryotic initiation factor 4E-binding protein (4E-BP), substrates of the TORC1 complex. PI3K and mTOR specific inhibitors can revert this hyperphosphorilation, suggesting the involvement of ataxin-2 in the PI3K/mTOR pathway (*Lastres-Becker et al., 2016*). Mice lacking these factors (RPS6 and 4E-BP) show hypoinsulinemia, glucose intolerance and reduction of adipose tissue (*Tsukiyama-Kohara et al., 2001; Ruvinsky et al., 2015*). On the contrary, ATXN2 KO mice are hyperinsulinemic, obese with increased abdominal adipose tissue accumulation (*Lastres-Becker et al., 2008b*), which

might be due to the hyperactivation of RPS6 and 4E-BP in the absence of ataxin-2. Moreover, it was shown that ataxin-2 expression is induced by starvation and by the mTOR inhibiton through rapamycin (*Lastres-Becker et al., 2016*). These results corroborate the suggestion that ataxin-2 acts as a metabolic sensor through mTOR signalling.

The fact that ataxin-2 achieves mTOR inhibition through stress granules formation suggests that this mechanism of cellular stress response could change metabolic regulation. In fact, stress granules have been discussed in the context of disease, namely in cancer, in light of its ability to sequester TORC1 (*reviewed in Heberle et al., 2015*). Regarding metabolic dysfunctions, such as diabetes or obesity, not much is known on stress granules role. The *Tia-1*- KO mice, where stress granules formation is impaired, have enhancement of several transcripts involved in lipid transport, tipically induced by fasting (*Heck et al., 2014*). Moreover, it is important to highlight that other stress granules components, such as TDP-43 and ataxin-2, are also involved on lipid metabolism, adipose tissue deposition and body weight regulation (*Stallings et al., 2013; Lastres-Becker et al., 2008b*; *Fittschen et al., 2015; Meierhofer et al., 2016*). A study by Cai and co-workers in 2014, observed, for the first time, a direct contribution of stress granules in a metabolic dysfunction setting (*Yan et al., 2014*). In this work, authors show that hypothalamic inflammation, that occurs in obesity, might be caused by a mechanism resulting from RNA stress granules formation. In this study, the authors show that obese mice have higher levels of TGF-β1, that promotes the sequestration of IκBα mRNA to stress granules blocking its translation, resulting in NF-κB activation. Moreover, stress granules genes were upregulated in the hypothalamus of high fat diet-fed mice and also in TGF-β1 treated mice (*Yan et al., 2014*). The results presented also suggest that stress granules are mediators of hypothalamic inflammation characteristic of obesity.

Given these findings and the fact that stress granules can modulate the main nutrient-sensing player, mTOR, it is of the utmost importance to look at stress granules as mediators of metabolic (dys)function and to understand the exact role of ataxin-2 in this context (Figure 10).

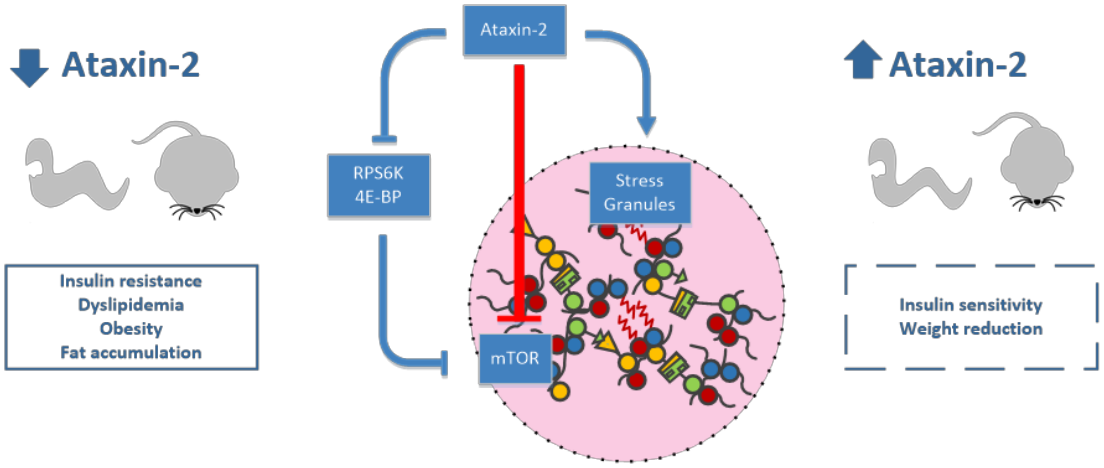

Figure 10: Ataxin-2 regulation of metabolic pathways – Stress granules mediated? Ataxin-2 deficiency has been correlated with metabolic dysfunction such as insulin resistance and dyslipidaemia in mice, leading to obesity and abnormal fat accumulation in mice and worms. Since this protein has been reported to modulate the mTOR pathway, it is possible that the metabolic actions of ataxin-2 might be mediated more or less directly through this pathway. Ataxin-2, which is influenced by nutritional status such as starvation, is able to block mTOR by promoting the decrease in the phosphorilation of RPS6K and 4E-BP which in turn blocks it. On other hand, stress granules might sequester mTOR mRNA blocking its translation and consequent action. This sequestration can be influenced by ataxin-2 expression since it is a major modulator of stress granules formation. Taken in consideration that blocking transiently mTOR is one intervention known to have a positive metabolic impact, ataxin-2 upregulation and its consequent blockage of this pathway might be a key for body weight reduction and improved metabolic parameters. Ataxin-2 increase might improve insulin sensitivity and, therefore, could be considered as a potential target for therapeutic interventions in metabolic disorders. This is a model suggested by the authors, combining the information obtained in different systems. We propose this model based on evidences regarding ataxin-2 orthologs, Pbp1 (*Saccharomyces cerevisiae*), atx-2 (*Caenorhabditis elegans*) and atxn2 (*Mus musculus*).

1.3. Hypothalamus as regulator of body homeostasis

The hypothalamus is the brain region responsible for the maintenance of body homeostasis and acts as a control center for endocrine functions. Hypothalamus is located below the thalamus, posterior to the optic chiasm, surrounding the third ventricle and integrates signals from circulating factors (*Faouzi et al., 2007*) (Figure 11).

The hypothalamus is composed by several nuclei as a complex network of distinct neurons that contain and release specific neuropeptides, neurotransmitters and have specific receptors (*Everitt, and Hokfelt, 1990*).

Hypothalamus can be divided in three main regions: *i*) the periventricular region constituted by the preoptic area (POA), suprachiasmatic nucleus (SCN), paraventricular nucleus (PVN), arcuate nucleus (ARH) and the posterior nucleus; (*ii*) the medial region formed by the medial postoptic nucleus (PON), anterior hypothalamic nucleus (AHN), ventromedial nucleus (VMN), dorsomedial nucleus (DMN) and premammillary nucleus and (*iii*) the lateral hypothalamic area (LHA) formed by the lateral preoptic nucleus, lateral hypothalamic nucleus, tuberomammillary nucleus and supraoptic nucleus (reviewed in *Elizondo-Vega et al., 2015*). The median eminence (ME) is located in the middle-basal hypothalamus and in the third ventricle walls, making contact with cerebrospinal fluid (CSF) (*Peruzzo et al., 2000*) (Figure 11).

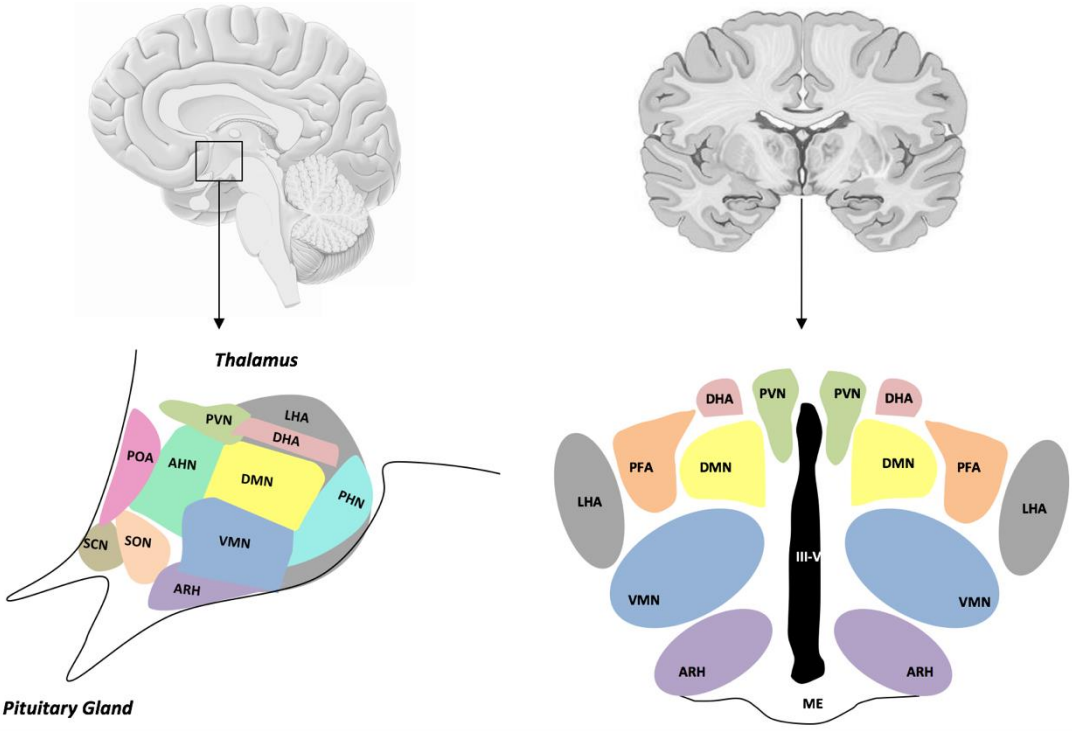

Figure 11. Anatomical localization of the hypothalamus and hypothalamic nuclei in human brain. (Left panel) Sagittal section near the midline of the human brain shows the hypothalamus localization (black box). Bellow, the schematic representation of the sagittal view of the distribution of hypothalamic

nuclei. (Right panel) Coronal section of the brain showing the hypothalamus along the third ventricle wall (black box). Bellow, the schematic representation of the coronal location of the hypothalamic nuclei. AN: arcuate nucleus; DHA: dorsal hypothalamic area; DMN: dorsomedial nucleus; LHA: lateral hypothalamic area; MB: mammillary bodies; ME: median eminence; PFA: perifornical area; POA: preoptic area; PVN: periventricular nucleus; SCN: suprachiasmatic nucleus; SON: supraoptic nucleus; VMN: ventromedial nucleus III-V: third ventricle. Adapted from (Elizondo-Vega et al., 2015)

1.3.1. Hypothalamic functions

Hypothalamic regulatory functions can be divided in three main categories: control of internal homeostasis, endocrine system regulation and autonomic nervous system (ANS) regulation. The regulation of internal homeostasis includes food intake, water balance, temperature, circadian rhythms and energy homeostasis. The endocrine system regulation mediated by hypothalamus is achieved via the pituitary gland, and regulates stress, growth, metabolism and reproduction. The autonomic nervous system regulation includes the control of blood pressure, gut motility and respiration (*Ganong, 2005; Mayer et al., 2009*). As the hypothalamus controls these different vital functions, the dysfunction of hypothalamic physiology can result in major health problems.

Hypothalamus has been long considered a key region in the pathogenesis of obesity and diabetes (*Gerozissis, 2008*), more recently it has also been correlated with the aging process (*Zhang et al., 2013; Yan et al., 2014; Cavadas et al., 2016*). Obesity and aging share several pathophysiological aspects and it was observed the disruption of hypothalamic functions in both of these conditions, where changes in circadian rhythms, stress response and energy balance occur (*Gerozissis, 2008*).

In this section we present and discuss the major physiological functions of the hypothalamus. We also highlight the contribution of hypothalamic regulation of peripheral organs and the impact of hypothalamic dysfunction in the overall body homeostasis.

1.3.1.1. Hypothalamus controls circadian rhythms

The earth rotation around its own axis and the consequent day and night cycling has major impact on the physiology of the organism. This rhythmicity dictates a need for adaption in order to maintain homeostasis. Circadian clocks (circadian deriving from the Latin *circa diem*, “about a day”) enable organisms to prepare for environmental changes such as light and temperature and adapt its behaviour accordingly, throughout the 24h of the day. Zeitgebers (the German word for “time givers”) are signals that control synchronization of body circadian clock (*Sahar and Sassone-Corsi, 2012; Gerber et al., 2015*). Circadian rhythmicity can be

observed in several physiological processes such as body temperature, activity, sleep, heart rate, blood pressure and hormone and neurotransmitters secretion (*Hastings et al., 2008*). In higher animals, these signals are coordinated by a core circadian oscillator or master clock. It has been known for nearly half a century that lesions of the mediobasal hypothalamus cause loss of circadian rhythmicity of locomotor activity, feeding and drinking (*Richter, 1967*). However, it was only in 1972 that the biological master clock was positioned within the mammalian SCN of the hypothalamus (*Moore and Eichler, 1972; Stephan and Zucker, 1972*).

Under normal circumstances, the SCN is reset daily: during the day by light inputs from the retina, and during the dark cycle by melatonin secretion (*Cassone et al., 1986*). These timing signals keep the clock in synchrony with the external day–night cycle. All cells in different tissues of the body present internal clocks that are controlled by the SCN. The SCN drives rest-activity cycles and feeding-fasting rhythms that are Zeitgebers to peripheral tissues. Neurons of the SCN have autonomous rhythmicity, which turns this nucleus quite plastic and allows for different levels of synchronization according with different cues (*Dibner et al., 2010; O'Neill and Reddy, 2012; Rosenwasser and Turek, 2015*). SCN neurons project to other regions of the central nervous system, especially to other hypothalamic nuclei. Moreover, it communicates to the periphery through endocrine signals released by other tissues of the central nervous system and through the sympathetic and parasympathetic nervous system (*Abrahamson and Moore, 2006; Vujovic et al., 2008; Eckel-Mahan and Sassone-Corsi, 2013*).

Time keeping and circadian rhythmicity in the master clock and all other cellular clocks is ensured by a transcription-translation feedback system (*Jin et al., 1999; Reppert and Weaver, 2002*). Briefly, two-transcription factors Circadian locomotor output cycles kaput (CLOCK) and aryl hydrocarbon receptor nuclear translocator like (ARNTL1 or BMAL1) heterodimerize and bind to the target gene promoter driving the rhythmic expression of *Period* (*Per1, Per2 and Per3*) and *Cryptochrome* (*Cry1 and Cry2*). PER and CRY then complex and translocate to the nucleus to inhibit CLOCK: BMAL1-induced gene expression. This loop takes around 24h to be completed and happens in all types of cells, driving all different clocks (*Dardente and Cermakion, 2007; Brown et al., 2012*).

Environmentally induced circadian disruptions or even genetic abnormalities in the circadian clock machinery can directly impact determinant physiological functions such as sleep and metabolic homeostasis. The disruption of those functions is directly correlated with chronic debilitating disorders such as cardiovascular diseases, obesity, diabetes and cancer (*Nagai et al., 1994; Achermann and Borbely, 2003*; *Meier-Ewert et al., 2004*; *Froy, 2010; Sahar and Sassone-Corsi, 2012; Eckel-Mahan and Sassone-Corsi, 2013; Challet, 2015; Coomans et al.,*

2015).

1.3.1.2. Hypothalamus orchestrates stress response

Exposure to stress is part of life and the adaptation of organisms to different types of stress is determinant for survival. Stress can be defined as the disruption in homeostasis and well-being, or the simple anticipation to it. Chronic stress or the lack of adaptive response to stress, either by psychosocial or genetic impairments, can result in psychosomatic or psychiatric disorders, such as depression (*Juruena et al., 2004; Juruena, 2014*). The physiological response to stress involves two major components, the hypothalamic-pituitary-adrenocortical (HPA) axis and the sympathetic nervous system (*Ulrich-Lai and Herman, 2009*).

The HPA-axis is activated following exhaustion, loss of control or the perception of some type of stress (*Ulrich-Lai and Herman, 2009*). The physiologic response to stress is mediated by the PVN of the hypothalamus, namely by neurosecretory neurons localized in the medial parvocellular portion of the PVN. These neurons release corticotropin releasing hormone (CRH) and arginine vasopressin (AVP), into portal circulation through the median eminence (*Swanson and Sawchenko,1983*). Releasing of these hormones stimulates the anterior pituitary gland to release adrenocorticotropin (ACTH), which in turn acts on the inner adrenal cortex to initiate the synthesis and release of glucocorticoid hormones, namely cortisol (*Dallman and Jones 1973; Gallagher et al., 2008*).

The release of glucocorticoids can act on two different pathways, to prevent excessive reaction to stress or to prepare the body for the possibility of prolonged stress. Therefore, in one hand, by negative feedback glucocorticoids inhibit the ongoing activation of the HPA axis (*Belda et al., 2015*), on the other, circulating glucocorticoids promote mobilization of stored energy and potentiate numerous sympathetic nervous system (SNS) mediated effects, such as peripheral vasoconstriction in order to prepare for further stress (*Sapolsky et al., 2000; Ulrich-Lai and Herman, 2009; Frank et al., 2013; Belda et al., 2015*).

Several conditions directly affect hypothalamic function, the HPA axis and adaptive stress response. For instance, chronic stress promotes obesity, type 2 diabetes and related cardiometabolic complications (*Harrel et al., 2016*). Depression is also tightly correlated with the risk for the development of metabolic syndrome (*Kyrou and Tsigos, 2009*). Reciprocally, obesity induces an inflammatory state that can stimulate the stress system and cause maladaptation leading to disorders such as depression (*Marazziti et al., 2014*).

1.3.1.3. Hypothalamus regulates fertility and reproduction

The hypothalamic– pituitary–gonadal (HPG) axis is responsible for the integration of hormonal signals and the regulation of reproduction. The HPG axis response includes the gonadotropin-releasing hormone (GnRH) neurons, which are mainly located at preoptic anterior hypothalamus (PAH) and project to the median eminence (*Prevot et al., 2010; Rudolph et al., 2016*). GnRH release in the pituitary stimulates the production production of follicle-stimulating (FSH) and luteinizing hormones (LH) that are released into the bloodstream. In the ovary, LH and FSH promote estradiol and progesterone secretion. At the testis, LH and FSH exert their effects on Leydig and Sertoli cells, respectively (*Schwanzel-Fukuda et al., 1992; Simoni et al., 1997; Chrousos et al., 1998; Dufau, 1998; Rudolph et al., 2016*).

GnRH neurons are regulated through autocrine mechanisms, by extracellular signals as neurotransmitters, steroid hormones and peptide hormones, and by paracrine inputs (*Muroi and Ishii, 2016*). These neuropeptides also influence sexual behaviour, namely neuropeptides involved in food intake regulation such as ACTH, alpha-melanocyte-stimulating hormone (α-MSH) and neuropeptide Y (NPY) (*Gonzalez et al., 1996; Muroi and Ishii, 2016*).

Obesity, and its metabolic consequences such as insulin resistance, but also anorexia reduce fertility and compromise pregnancy, both in humans and animal models (*Brüning et al., 2000; Tortoriello et al., 2004; Seli et al., 2014; Tsatsanis et al., 2015; Clarke and Arbabi, 2016*). The connection between energy balance and fertility might derive from an evolutionary strategy.

1.3.1.4. Hypothalamus dictates energy balance regulation

Hunger is defined as the necessity or desire for food, while satiety results from being satisfied. Feeding gives the energy required for the maintenance of physiological homeostasis. A state of energy homeostasis results from the balance between energy intake and energy expenditure. This equilibrium might be modulated by peripheral signals reflecting body energetic supplies, and also by environmental and behavioural aspects that can change body weight (*Webber et al., 2015; Benite-Ribeiro et al., 2016*). Peripheral organs, such as the stomach, gut, pancreas and adipose tissue, are responsible for producing peripheral hormones that reach the hypothalamus in order to maintain energy balance (*Ueno and Nakazato, 2016*). Besides the diverse nuclei of the hypothalamus, several other brain areas are involved in the regulation of energy balance, such as the nucleus tractus solitaries (NTS) of the brainstem, the paraventricular thalamus (PVT), the parabrachial nucleus (PBN), the ventrolateral periaqueductal gray and dorsal raphe complex (PAGvl/DR) and the ventral tegmental area

(VTA) (*Waterson and Horvath, 2015*) (Figure 12).

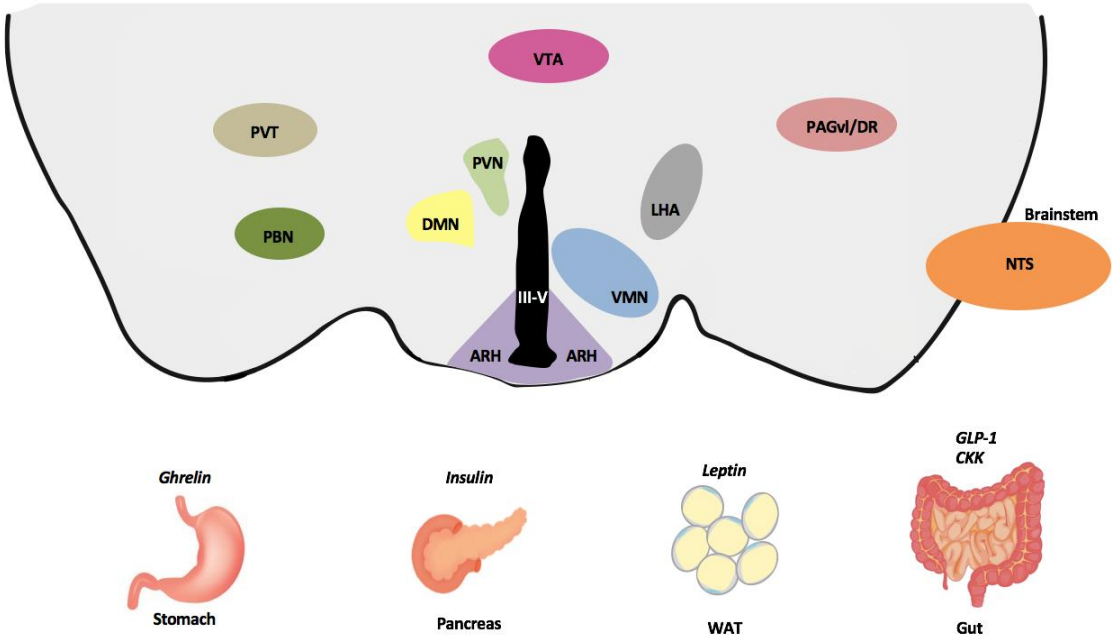

Figure 12. Brain areas and peripheral organs involved on energy balance regulation. Peripheral organs such as the stomach, pancreas, white adipose tissue (WAT) and gut release hormones or peptides that act on the arcuate nucleus of the hypothalamus (ARH) and activate response to balance food intake and energy expenditure. Other brain regions such as paraventricular thalamus (PVT), the parabrachial nucleus (PBN), the ventral tegmental area (VTA), the ventrolateral periaqueductal gray and dorsal raphe complex (PAGvl/DR) and the brainstem are also involved in the regulation of energy balance. The hormones released by the gut during food digestion, act mainly on the brainstem to inhibit food intake. Moreover, glucagon-like peptide 1 (GLP-1) has an incretin effect which means that increases insulin release, promoting food intake inhibition and energy expenditure. ARH, arcuate nucleus; CKK, cholecystokinin; DMN, dorsomedial nucleus; LHA, lateral hypothalamic area; NTS, nucleus tractus solitarius; PAGvl/DR, ventrolateral periaqueductal gray and dorsal raphe complex; PBN, parabrachial nucleus; PVN, paraventricular nucleus; PVT, paraventricular thalamus; VMN, ventromedial nucleus; VTA, ventral tegmental area; III-V: third ventricle. Adapted from (Waterson and Horvath, 2015).

The hypothalamus is the main sensory brain region to regulate hunger and feeding (*Hetherington and Ranson, 1940, 1942*). As a homeostatic energy sensor, hypothalamus detects peripheral signals reflecting the nutritional and metabolic status and integrates this information (*Blouet and Schwartz, 2010*). Hypothalamic neurons then respond by synthesizing and releasing neuropeptides that exert orexigenic (food intake stimulation) or anorexigenic (food intake inhibition) effects (*Kalra and Kalra, 2010; Williams and Elmquist, 2012; Waterson and Horvath, 2015*).

Hypothalamus has several nuclei (ARH, PVN, VMN, DMN, LHA) involved in energy balance regulation. The first study that related hypothalamus and food intake showed that lesions in this brain region affected hunger and satiety (*Hetherington and Ranson, 1940*). Moreover,

lesions in rat VMN increased feeding, whereas lesions in VLH decreased feeding (*Anand and Brobeck, 1951a, 1951b*). These observations suggested that hypothalamic nuclei might play different roles in food intake regulation.

The ARH, localized within the diencephalon along the third ventricle near the median eminence, is the critical energy balance sensor (*Broadwell and Brightman, 1976*). The blood brain barrier at the median eminence is fenestrated so circulating hormonal, metabolic and nutritional signals easily reach ARH neurons (*Schwartz et al., 2000; Myers et al., 2009; Langlet, 2014*). The ARH has two distinct populations of neurons: the orexigenic neurons that produce and release NPY and Agouti-Related- Protein (AgRP); and the anorexigenic neurons that produce and release pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART). AgRP/NPY neurons stimulate food intake while POMC/CART decrease food intake, promoting satiety and energy expenditure (*Hahn et al., 1998; Schwartz et al., 2000*). In a normal physiological state, the appropriate functioning of these two populations of neurons in response to peripheral hormones, ensures the balance between food intake and energy expenditure (*Williams et al., 2001; Kalra and Kalra, 2010*).

For example, cells in gastric epithelium produce and release ghrelin that will reach the ARH, activating ghrelin receptors located on NPY/AgRP neurons that will stimulate NPY and AgRP release, thus promoting food intake (*Kamegai et al., 2000; Betley et al., 2015*). Plasmatic ghrelin levels increase just before a meal and rapidly decrease after food intake (*Tschop et al., 2000; Ariyasu et al., 2001, Cummings et al., 2001*). Upon food intake, leptin will be synthesized and secreted from adipose cells and insulin from pancreatic Beta cells, leading to compensatory inhibition of NPY/AgRP neurons and activation of POMC/CART neurons, thus decreasing food intake (*Barsh and Schwartz, 2002; Friedman, 2002*). Concentrations of circulating insulin and leptin are proportional to the body adiposity (*Bagdade et al., 1967; Considine et al, 1996*), therefore, when energy storage is low (or in fasting), the circulating levels of insulin and leptin decrease, thus activating the orexigenic NPY/AgRP neurons while inhibiting anorexigenic POMC/CART neurons. This effect of peripheral signals in the activation/inhibition of ARH neurons is responsible for the maintenance of body weight and energetic homeostasis (*reviewed in Kim et al., 2014; Webber et al., 2015; Waterson and Horvath, 2015*) (Figure 13).

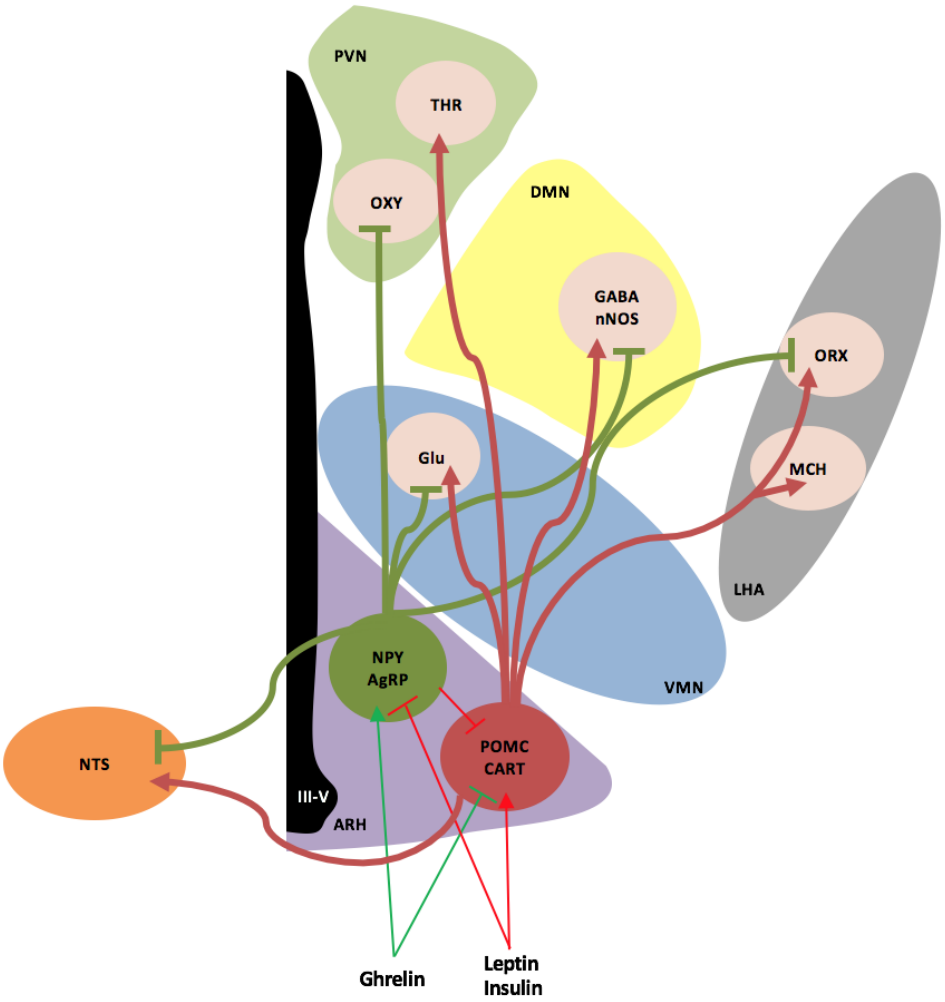

Figure 13. Hypothalamic neural circuitry responsible for food intake and energy balance. Arrows represent activating inputs and blunt-ended lines represent inhibitory inputs. Green represents connections that promote food intake and decrease energy expenditure, whereas red represents connections that inhibit food intake and promote energy expenditure. Ghrelin released from the stomach stimulates NPY/AgRP neurons and inhibits POMC/CART neurons, consequently stimulating food intake. The activation of NPY/AgRP neurons stimulates the release of NPY and AgRP that promote an orexigenic effect; moreover, the release of NPY and AgRP inhibits POMC/CART neurons in the arcuate nucleus of the hypothalamus (ARH). NPY/AgRP neurons projections inhibit neurons located in other hypothalamic nuclei, namely in the paraventricular nucleus (PVN), the ventromedial nucleus (VMN), the dorsomedial nucleus (DMN) and the lateral hypothalamic area (LHA). NPY/AgRP neurons also project to the brainstem to further promote food intake and to suppress brown adipose tissue (BAT) thermogenesis. Insulin and leptin stimulate POMC/CART neurons and inhibit NPY/AgRP neurons, therefore inhibit food intake and promote energy expenditure. POMC/CART neurons release alpha-melanocyte stimulating hormone (α-MSH) that has anorexigenic effect. POMC/CART neurons also innervate other nucleus and have a mostly excitatory input in the neurons in VMN, DMN, PVN and LHA, to ensure a decrease in energy intake. POMC/CART neurons also innervate the nucleus tractus solitarius (NTS) of the brainstem, where the release of α-MSH potentiates the effect of the satiety signals released by the gut, cholecystokinin (CCK) and glucagon-like peptide 1 (GLP-1). CRH, Corticotrophin-releasing hormone; GABA Gamma-Aminobutyric acid; MCH, Melanin-concentrating hormone; nNOS, neuronal Nitric oxide synthases; ORX, Orexins; TRH, Thyrotropin-releasing hormone; III-V: third ventricle. Adapted from (Mercer et al., 2011; Waterson and Horvath, 2015).

1.3.2. Hypothalamic autonomic control of body energy homeostasis

Central nervous system can receive and integrate signals relative to energetic status from the periphery and orchestrate a response by the modulation of food intake and energy expenditure. The hypothalamus, as the brain region responsible for the balance between food intake and energy expenditure, is involved in the control of major metabolic organs likes the liver, white adipose tissue (WAT), brown adipose tissue (BAT) and pancreas (Figure 14). This regulatory role of the hypothalamus over the periphery is obviously intimately connected to the regulatory mechanisms of food intake and energy expenditure.

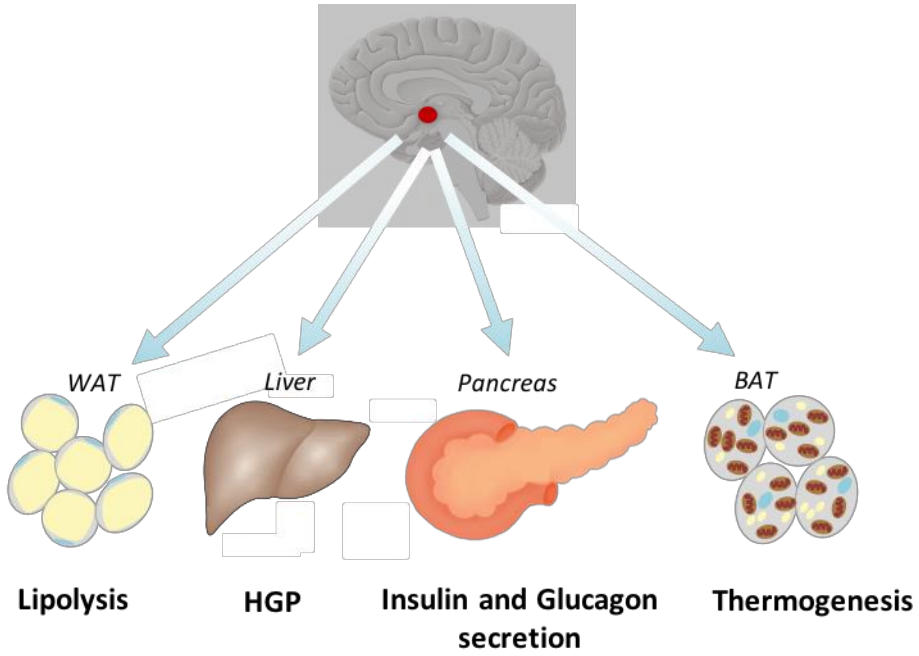

Figure 14: Autonomic control of metabolic homeostasis. Metabolic homeostasis is controlled by the central nervous system (CNS) that receives and integrates signals form peripheral tissues and the external environment. The CNS controls autonomic regulation of peripheral organs through several areas, namely the hypothalamus. The hypothalamus regulates white adipose tissue (WAT) lipolysis through the LHA, VMN and ARH. Glucose homeostasis is regulated through sympathetic and parasympathetic innervation of the liver and pancreas. The LHA, VMN, PVN and ARH are involved in the hepatic glucose metabolism, specially in the regulation of hepatic glucose production (HPG). Hypothalamic modulation of pancreatic secretion of insulin and glucagon is achieved mainly through the VMN, DMV and ARH. The VMN, DMN and PVN are directly related with the stimulation of thermogenesis in BAT.

The liver is a key organ in energy storage and in the regulation of glucose homeostasis. The liver receives fatty acids through hydrolysis of triglycerides coming from the gut after a meal and non-esterified fatty-acids from lipolysis of WAT during fasting. Furthermore, the liver can synthetize fatty acids from excess glucose in a process called *de novo* lipogenesis. Fatty acids then can be used by the liver for oxidation, assembled into triglycerides and stored in the liver

or secreted into the plasma in very-low-density lipoproteins (VLDL) particles (*Gibbons, 1990; Mason, 1998; Nguyen et al., 2008; Bruinstroop et al., 2014*). The ARH is directly involved in the secretion of VLDL-TG; in one hand NPY stimulates VLDL-TG secretion, whereas POMC blockade does not change VLDL-TG (reviewed in *Bruinstroop et al., 2014*). However, chronic blockade of melanocortin system in hypothalamic neurons can promote lipogenesis and lipid accumulation in the liver (reviewed in *Bruinstroop et al., 2014*). In fact, melanocortin 4 receptor (MC4R) knockout mice have increased levels of fatty acid synthase (FAS) and show hepatic steatosis (*Albarado et al., 2004*).

The liver is also involved in insulin sensitivity, and responds to glucose and insulin by modulating hepatic glucose production (HGP) (*Rammanon et al., 2011*). Several hypothalamic nuclei have been implicated in the regulation of HGP. VMN stimulation promotes hepatic glucose output and decreases glycogen in the liver (*Shimazu, 1979*), whereas LH stimulation increases hepatic glycogen synthesis (*Shimazu et al., 1975*). The ARH receives insulin, leptin and glucose from the periphery and gives signals to the PVN for glucose homeostasis regulation. POMC/CART neurons major product, α MSH, does not change the inhibitory effect of hypothalamic insulin on HPG, however leptin-induced responses appear to depend on POMC neurons. The ablation of leptin receptor in POMC neurons increases HGP (*Parton et al., 2007; Bisschop et al., 2015*). NPY/AgRP neurons are also essential for leptin-induced reduction of glucose, however an intracerebroventricular (ICV) injection of NPY leads to the increase in glucose production (*Coppari et al., 2005; Van den Hoek et al., 2008; Seoane-Collazo et al., 2015*).

BAT has thermogenic function, promotes energy expenditure and is profoundly affected upon certain diets (*Whittle et al., 2011; Whittle et al., 2012; Contreras et al., 2014; Morrison et al., 2014*). The VMN, DMN and the ARH are directly connected to BAT activity (*Seoane-Collazo et al., 2015*). In these structures, leptin sensing and signalling pathways involved in nutrient detecting, such as AMPK (5' AMP-activated protein kinase), play a determinant role in BAT activation. AMPK activation in the VMN is a negative regulator of BAT activation (*Lopez et al., 2010; Whittle et al., 2012; Tanida et al., 2013*), while leptin in the DMN mediates thermogenesis, increasing energy expenditure. The NPY knockdown in the DMN also promotes energy expenditure and induces WAT “browning” (*Chao et al., 2011*). In the ARH, leptin receptors are determinant for BAT activation. Leptin enhances the excitability of POMC/CART neurons that are directly responsible for the promotion of thermogenesis, while inhibiting NPY/AgRP neurons that prevent thermogenesis (*Harlan et al., 2011; reviewed in Morrison et al., 2014*).

WAT stores energy in form of triglycerides, but also releases several hormones/adipokines that regulate glucose and lipid metabolism (*Ahima and Flier, 2000*). Metabolic pathways in adipocytes determine the release of triglycerides into circulation or its accumulation in certain tissues like in the liver (*Guilherme et al., 2008*). The sympathetic nervous system regulates lipolysis, adipocyte number and WAT proteins (*Nogueiras et al., 2010*). The hypothalamus as a regulator of food intake and overall energetic balance, is also involved in WAT regulation. The ARH, DMN, LHA, PVN and SCN have all been related to WAT function (*Bamshad et al., 1998*). The activation of neurons of VMN and of LHA can promote lipolysis (*Ruffin and Nicolaidis, 1999; Shen et al., 2008; Perez-Leighton et al., 2014*). As expected, leptin also plays a role in WAT. In rats MBH, the injection of leptin inhibits lipogenesis (*Buettner et al., 2008*) and when administered in the third ventricle, leptin increases lipolysis in WAT (*Tajima et al., 2005*). In the ARH, NPY promotes energy storage in WAT, increasing adiposity (*Baran et al., 2002; Sousa-Ferreira et al., 2012*). Accordingly, a decrease of α-MSH (product of POMC/CART neurons) also increases adiposity and impairs lipolysis (*Kaushik et al., 2012*).

The hypothalamus also regulates pancreatic insulin production and skeletal muscle glucose uptake. VMN glucose sensing neurons increase glucagon secretion upon high glucose levels (*Borg et al., 1995, 1997*). The reduction of insulin receptors in the neurons of VMN impairs pancreatic cells function (*Paranjape et al., 2011; Seoane-Collazo et al., 2015*). The direct stimulation of VMN and/or the combined action of leptin and orexin in this nucleus, stimulates glucose uptake by the skeletal muscle (*Shimazu et al., 1991; Sudo et al., 1991; Shiuchi et al., 2009; Seoane-Collazo et al., 2015*).

Hypothalamus integrates peripheral signals and modulates several peripheral organs to maintain energy balance. Therefore, alterations in the hypothalamus will change the tightly controlled energy homeostasis. Also, changes in diet alter hypothalamic physiology and may disrupt the peripheral organs regulation, which will result in a vicious cycle of metabolic instability.

1.3.3. Hypothalamic dysfunction in metabolic disorders

The complexity of energy balance regulation and the determinant role of the hypothalamus, raised several questions regarding the control of metabolic dysfunction in this brain region. Hypothalamic circuits are strongly regulated to prevent severe changes in body weight and energy homeostasis. However genetic and environmental factors can overcome this regulatory effects and promote severe body weight alterations.

Overnutrition is one of the main causes for obesity (*Kolb et al., 2010; Williams et al., 2012*). It is now accepted that overnutrition and consequent obesity are characterized by a low-grade systemic inflammation (*Hotamisligil et al., 2006; Kolb et al., 2010; Odegaard and Chawla, 2013*). The activation of inflammatory pathways can affect peripheral tissues like liver, BAT, WAT and skeletal muscle (*Cai et al., 2005; Itani et al., 2005; Bandyopadhway et al., 2005*). Hypothalamic inflammation is now also considered a feature of obesity pathophysiology (*DeSouza et al., 2005; Zhang et al., 2008; Posey et al., 2009*). Hypothalamic inflammation was observed in one day after consumption of a high-fat diet and can promote severe cellular damages such as apoptosis (*Thaler et al., 2012; Waise et al., 2015*). In this context, it is possible to observe inflammation in the mediobasal hypothalamus, specially in ARH and median eminence (*Hotamisligil et al., 1993; Uysal et al., 1997; Hundal et al., 2002; Elizondo-Vega et al., 2015*). In fact, an acute increase in glucose or lipids in the third ventricle are enough to increase pro-inflammatory molecules in the hypothalamus (*Zhang et al., 2008; Milanski et al., 2009*). The major problem related to hypothalamic inflammation is the fact that it persists even after weight loss and increases the predisposition to further weight re-gain (*Wang et al., 2012*). This observation suggests that the hypothalamic damage induced by a high-fat diet could be irreversible (*Moraes et al., 2009; Van de Sande-Lee et al., 2011; Thaler et al., 2012*). Hypothalamic inflammation is prior to the onset of obesity and the inflammation in peripheral tissues. Several studies showed that hypothalamic inflammation can in fact be the cause for the overall metabolic alterations observed in obesity (*Valdearcos et al., 2015*).

Hypothalamic inflammation can impair the normal regulation of peripheral organs. Hypothalamic inflammation was already related to increased blood pressure by the inhibition of POMC neurons (*Purkayastha et al., 2011*). Insulin release and action can be impaired by the compromise of the connection between pancreas and hypothalamus. Hypothalamic inflammation may induce insulin resistance, independently of body weight increase (*Kang et al., 2009; Calegari et al., 2011*). Thermogenic ability of BAT is also impaired upon inflammatory cytokines increase in the hypothalamus, like for example TNF-α. In fact, TNFα receptor type 1 (TNFR1) KO mice are protected against HFD-induced obesity and have increased BAT activation

(*Romanatto et al., 2008; Arruda et al., 2010; Arruda et al., 2011*). Hypothalamic pro-inflammatory state can also increase WAT adipocytes, WAT-inflammation and liver steatosis (lipid accumulation) (*Guilherme et al., 2008; Williams, 2012; Valdearcos et al., 2015*). Leptin and insulin resistance, promoted by hypothalamic inflammation, can impair the hypothalamic autonomic regulation over peripheral organs (*reviewed in Williams, 2012; Seoane-Collazo et al., 2015; Valdearcos et al., 2015*) (Table 5).

There are several potential cellular mechanisms underlying the hypothalamic inflammation caused by overnutrition. Briefly, loss of the neurogenic ability of the hypothalamus can aggravate the impairment in energy balance, creating a feedback loop where inflammation is further potentiated (*Lee et al., 2012; McNay et al., 2012*). Loss of proteostasis is tightly correlated with hypothalamic dysfunction and can be further promote inflammatory responses (reviewed in *Cavadas et al., 2016*). Mitochondrial dynamics, ROS excessive formation and impaired ROS signalling are also known to promote deep alterations in hypothalamic circuitry (reviewed in *Williams, 2012*). Autophagy is an important response to cellular stress, especially to ER stress and ROS, which are both enhanced by over-nutrition. The constant stress, impairs autophagy and consequently the cellular ability to remove cell damage (*Meng and Cai, 2011*). The accumulation of saturated fatty acids in the hypothalamus can also increase inflammation and neuronal dysfunction (*Posey et al., 2009; Martinez de Morentin et al., 2010*) (Table 5).

Even though hypothalamic inflammation mediates most of the pathophysiologic effects of overnutrition, this phenomenon can be a paradox. In one hand, evidences suggest that hypothalamic inflammation can also induce neuronal injury and neuronal plasticity impairment (*Lee et al., 2012; McNay et al., 2012*, *Thaler et al., 2012*), which contributes to obesity. On the opposite, in the context of systemic diseases such as cancer, hypothalamic inflammation is related to anorexia-cachexia syndromes (*Sherry et al., 1989; Laviano et al., 2000; Pimentel et al., 2014*).

Some studies suggest that blocking inflammatory processes in metabolic disorders improves insulin sensitivity and lowers the risk of type 2 diabetes and cardiovascular diseases (*Yuan et al., 2001; Sauter et al., 2008; Osborn et al., 2008; Larsen et al., 2007; Zhang et al., 2008; Stanley et al., 2011*). Given the regulatory role of the hypothalamus over global energy homeostasis it is not surprising that blocking pro-inflammatory pathways in the hypothalamus is more beneficial to the metabolic status, than specifically blocking inflammation in key metabolic organs.

Table 5. Effect hypothalamic inflammation on different tissues and organs

Tissue/Organ/System	***Effect of hypothalamic inflammation***	***References***
WAT	White adipocyte enlargement WAT inflammation	*Kim et al., 2007; Wang et al., 2011*
BAT	Reduced thermogenesis	*Arruda et al., 2011; Xu et al., 2016*
Liver	Hepatosteatosis Deregulation of HGP	*Lam et al., 2007; Arruda et al., 2011; Milanski et al., 2012*
Pancreas	β cells dysfuntion- reduced insulin production	*Calegari et al., 2011*
Cardiovascular system	Increased blood pressure	*Kang et al., 2009; Purkayastha et al., 2011*
Hormones	Insulin and leptin resistance	*Carvalheira et al., 2003; De Souza et al., 2005; Enriori et al., 2007; Kang et al., 2009; Kleinridders et al., 2009; Ropelle et al., 2010; Calegari et al., 2011*
Hypothalamic neuronal circuitry	Apoptosis Disruption of neurogenesis Deregulation of energetic balance	*Zhang et al., 2008; Moraes et al., 2009; Thaler et al., 2010; Purkayastha et al., 2011; Thaler et al 2012; Lee et al., 2012; McNay et al., 2012; Sousa-Ferreira et al., 2014;*
Cellular mechanisms	ER stress ROS and mitochondrial dysfunction Autophagy impairment	*Lowell and Shulman, 2005; Matsuzawa-Nagata et al., 2008; Zhang et al., 2008; Ozcan et al., 2009; Denis et al., 2010; Kaushik et al., 2011; Meng and Cai, 2011; Valdearcos et al., 2015; Cavadas et al., 2016*

1.4. Ataxin-2 as a player in hypothalamic physiology

Ataxin-2 has a widespread expression in adult and embryonic tissues, predominantly in the brain, but also in the heart, muscle, intestine, liver and lung (*Pulst et al., 1996*). As also mentioned before, ataxin-2, as well as its interactor protein A2BP1 (an obesity-related gene) are expressed in the hypothalamus (*Nechiporuk et al., 1998; Ma et al., 2010*). The observations in the ATXN2 KO mice were mainly relative to alterations in fertility and body weight and metabolism, two main physiological functions controlled by the hypothalamus. To date there are no studies regarding ataxin-2 role in hypothalamic physiology, however there are some information that might support that ataxin-2 may have a relevant role in hypothalamus.

Given the hypothalamic role in body weight and energetic balance (*See section 1.3.1.4 and 1.3.2*) and all the evidences showing ataxin-2 as a metabolic mediator (*See section 1.2*) it is easy to conceive a role for ataxin-2 in hypothalamic physiology. The modulatory effect of ataxin-2 over metabolic signalling pathways such as mTOR and AMPK (*See section 1.2.4*), provides a possible mechanism through which ataxin-2 might play an overall catabolic role in energy balance.

Circadian rhythm regulation is closely related with metabolism (*Eckel-Mahan and Sassone-Corsi, 2013; Challet, 2015; Coomans et al., 2015*). The impact of ataxin-2 in body homeostasis might also result from a role in circadian rhythm regulation. Studies in *Drosophila* involved ataxin-2 in the translation of circadian clock genes (*Lim et al., 2011; Lim and Allada et al., 2013; Zhang et al., 2013*). The *twenty-four* (*tyf*) gene binds directly to ataxin-2 to regulate PERIOD (PER) translation, thus regulating dark/light period in Drosophila (*Lim et al., 2011; Zhang et al., 2013*). Furthermore, a depletion in ataxin-2 resulted in a long-period of constant dark and the re-establishment of ataxin-2 levels, restored the normal circadian rhythm (*Lim and Allada, 2013*).

Sleep patterns and circadian rhythms are tightly connected. Individuals with SCA2 show disturbed rapid eye movement (REM) sleep, restless leg syndrome and daytime sleepiness even before the onset of ataxia symptoms (*Tuin et al., 2006; Boesch et al., 2006; Rodríguez-Labrada et al., 2011; Pedroso et al., 2016*). Alterations in sleep patterns in SCA2 patients might arise from disturbed circadian rhythms due to ataxin-2 mutation.

ATXN2 KO mice show hyperactivity and reduced response facing fear (*Huynh et al.,* 2009), implying that ataxin-2 might have a role in stress response regulation. One of the major ataxin-2 interactor, A2BP1, was found to be upregulated during stress and to be required for the CRH transcription and the consequent activation of the HPA axis (*Amir-Ziberstein et al., 2012*).

A2BP1 is able to modulate ataxin-2 expression (*Ma et al., 2010*), hence its increase might result in a concomitant increase of ataxin-2 during stress. Moreover, A2BP1 and ATXN2 were already associated with psychiatric disorders such as bipolar disorder, dementia and schizophrenia (*Hamshere et al., 2009; Le-Niculescu et al., 2009; Davis et al., 2012; Zhang et al., 2014*). At cellular level, ataxin-2 also mediates stress response, promoting the formation of stress granules (*See 1.1.1.5.4*). There are no reports regarding stress granules formation and psychiatric disorders. However, it is easy to conceive that if at cellular level, stress is not overcome, this will reflect in the functioning of the HPA axis and therefore in the overall stress response. Alterations in ataxin-2 and its interactors could promote the abnormal activation of the HPA axis and inappropriate stress response, hence leading to behavioural/psychiatric alterations.

Fertility regulation is also a major physiologic function of the hypothalamus, that is also deeply affected by the metabolic status of the organism (*Brüning et al., 2000*). The knockout of ataxin-2 in C. elegans and mouse promoted a deviation from the sex ratio, with increased number of males (*Ciosk et al., 2004; Kiehl et al., 2006; Lastres-Becker et al., 2008b*). ATXN2 KO mice were found to be fertile, however with a lower number of animals per litter suggesting that ataxin-2 might have a role in fecundity/fertility (*Kiehl et al., 2006; Lastres-Becker et al., 2008b*). ATXN2 KO mice are also insulin resistant which has been also related with reproductive abnormalities (*Brüning et al., 2000*). Even though the effect of absence and/or overexpression of ataxin-2 on fertility and reproduction is poorly studied and understood, evidence suggests that ataxin-2 fluctuations might promote fertility abnormalities.

This compelling list of evidences definitely correlates ataxin-2 with hypothalamic physiology. A better understanding of ataxin-2 role in the hypothalamus would not only improve our understanding of the hypothalamic regulation of body homeostasis, but also provide a new target for therapeutic strategies for disorders that promote hypothalamic dysfunction such as obesity.

CHAPTER 2

Objectives

2. Main objectives

The hypothalamus, namely the hypothalamic arcuate nucleus (ARH), receives and integrates peripheral hormonal and nutritional signals through two neuronal populations (CART/POMC expressing neurons and AgRP/NPY expressing neurons) regulating appetite, food intake and energy expenditure (*reviewed in Kim et al., 2014; Webber et al., 2015; Waterson and Horvath, 2015*). The knowledge of molecular components of these two neuronal populations contributes to a better understanding of molecular mechanisms of obesity pathophysiology and to the development of new putative therapeutic approaches.

Ataxin-2 is encoded by ATXN2 gene located at 12q23 – 12q24.1. This gene is part of the *ATXN2/SH2B3* trait, highly related with risk for obesity and other metabolic dysfunctions (*Auburger et al., 2014*). Moreover, ATXN2 knockout mice are obese and insulin resistant, presenting severe compromise in lipid metabolism (*Kiehl et al., 2006; Lastres-Becker et al., 2008b; Meierhofer et al., 2016*). Ataxin-2 is also correlated with insulin sensitivity, through its actions in the endocytosis of tyrosine kinase receptors (*Nonis et al., 2008; Drost et al., 2013*) and its modulator potential of the mTOR pathway (*Takahara and Maeda, 2012; DeMille et al., 2015; Bar et al., 2016; Lastres-Becker et al., 2016*). All these evidences suggest that ataxin-2 could be a metabolic mediator (*Carmo-Silva et al., 2017*) but the potential role of ataxin-2 on the hypothalamic regulation of energy homeostasis regulated is not known.

Taking all this information into account, the main objectives of the present work are the following:

- To study the impact of metabolic challenge, such as high-fat diet (HFD), on the hypothalamic ataxin-2 expression.
- To investigate whether ataxin-2 silencing in the ARH promotes metabolic dysfunction.
- To investigate the potential of ARH ataxin-2 levels overexpression as an anti-obesity strategy.
- To investigate the role of hypothalamic ataxin-2 in the overall metabolic status regulation.

CHAPTER 3

Materials and Methods

3.1. *In vivo* studies

3.1.1. Animals

For experiments in Chapter 4 and 5, adult C57BL/6J mice were purchased from Charles River Laboratories (Wilmington, MA, USA). Mice were housed 2-3 *per* cage under a 12 h light/ dark cycle in a temperature/humidity controlled room with *ad libitum* access to water and food. For the experimental procedures standard chow diet and Rodent Diet with 45% kcal% fat (D12451 from Research Diets, NJ, USA) were used.

For experiments in Chapter 6, C57Bl/6x129SvJ background ATXN2 KO mice ($SCA2^{-/-}$), heterozygous ($SCA2^{+/-}$) and wild-type ($SCA2^{+/+}$) littermates were kindly provided by Prof. Stefan M. Pulst (University of Utah, UT, USA) for this study. Mice were housed 2-3 *per* cage under a 12-h light/ dark cycle in a temperature/humidity controlled room with *ad libitum* access to water and food. To recapitulate previous studies with this strain (*Keihl et al., 2006; Scoles et al., 2012*), all the experimental procedures were performed in mice fed with Rodent Diet with 45% kcal% fat (D12451 from Research diets).

Experiments were performed in accordance with the European Union Directive 86/609/EEC for the care and use of laboratory animals. Researchers received adequate training (Federation of Laboratory Animal Science Associations (FELASA) certified course) and certification to perform the experiments from the Portuguese authorities (Direção Geral de Veterinária). In addition, animals were housed in our licensed animal facility (International Animal Welfare Assurance number 520.000.000.2006).

3.1.2. Production of Viral vectors

The cDNA encoding for GFP (*Nobrega et al., 2013*), ShAtx2 (MISSION® shRNA, Sigma-Aldrich, MO, USA) and human Atx2 (ATXN2) with 23 CAG (GC-Y4388, Genecopoeia), were cloned in a self-inactivating lentiviral vector as described previously (*Deglon et al., 2000*). All the viral vectors encoding for the different constructs were produced in human embryonic kidney (HEK) 293T cells using a four-plasmid system described previously (*de Almeida et al., 2002*). Viral titer was assessed by quantification of p24 by enzyme-linked immunosorbent assay (HIV-ELISA, Zeptometrix, NY, USA). Viral stocks were stored at -80 °C until use.

3.1.3. Stereotaxic injection of lentiviral vectors

For the stereotaxic injection of lentiviral vectors, concentrated viral stocks were thawed on ice and resuspended by vortexing. Mice with 8 weeks old were anaesthetized with ketamine (75 mg/kg intraperitoneal administration, IP) and xylazine (10 mg/kg IP). Particle content of lentiviral vectors was matched to 200 000 ng of p24/mL. The ARH was defined by using The Paxino's Mouse Brain Atlas, as performed in previous studies (*Aveleira and Botelho et al., 2015*). Injections were performed bilaterally into the ARC: 0.5 mm lateral to the middle line, 1.65 mm posterior to the bregma and −5.8 mm ventral to the brain surface. Mice received a single injection of lentivirus encoding for either for GFP, ShAtaxin-2 or Ataxin-2 according with the experiment, in the ARH of each hemisphere in a final volume of 2 µL. Injection was performed at a rate of 0.5 µL/min with a 10 mL-Hamilton syringe attached to an automatic Pump Controller (World Precision Instruments, FL, USA). Needle was kept in place for 5 min to minimize backflow. Mice were allowed to recover for two days before body weight and food intake analysis.

3.1.4. Food intake and body weight analysis

For experiments in Chapter 4 and 5, mice were housed 2-3 *per* cage and monitored for 4 weeks after lentiviral vectors injection. Mice were kept either in chow or high fat diet in a total of 4 groups (Chapter 4: Chow Control (GFP), Chow ShAtx2; HFD Control (GFP), HFD ShAtx2; Chapter 5: Chow Control (GFP), Chow Ataxin-2; HFD Control (GFP), HFD Ataxin-2).

For experiments in Chapter 6, mice were housed 2-3 *per* cage and monitored for 8 weeks after lentiviral vectors injection. Mice were kept in high fat diet in a total of 4 groups (WT (wild-type), KO, KO+Ataxin-2, Het (heterozygous)).

For all experiments, body weight and food intake were measured twice a week. Body weight gain was calculated in % of weight gain and also presented in a plot graph as cumulative weight gain (g), since 2 days after stereotaxic injection (Day 0). Food intake was measured as a ratio of total food ingested over the study (g) per total weight gained (g).

3.1.5. Insulin tolerance test (ITT)

For ITT, mice were fasted for 6h and injected IP with 1 U/Kg of insulin (Lilly, IN, USA). Blood samples were collected at 0, 5, 10, 15, 20, 25 and 30 min from the tail for glucose measurement using a glucometer (FreeStyle Precision Neo from Abbott, IL, USA). The rate

constant for glucose clearance (k_{ITT}) was calculated using the formula $0.693/t_{1/2}$. The plasma glucose $t_{1/2}$ was calculated from the slope of the least squares analysis of the plasma glucose concentration during the linear phase of decline (5-15 min) (*Bonora et al. 1989*).

3.1.6. Behavioral assessment

For the assessment of mice locomotor horizontal activity, the Open field test was performed. For experiments in Chapter 5, Open field was performed once, 24 days after stereotaxic injections. For experiments in Chapter 6, Open field test was performed twice, 42 and 44 days after stereotaxic injections. The Open field test was performed the first time within the inactive period of the mice (3 h after the beginning of the 12 h light cycle) and the second time within the active period (3 h after the beginning of the 12 h dark cycle).

For all the tests, mice were acclimated into the test room for a 12 h period. Mice were placed in a 50×50 cm arena with 50 cm high walls and their movement activity was recorded for 40 min using the Acti-Track System (Panlab, Barcelona, Spain). The activity tracing of the two zones of the box, and the mean values for total distance travelled and velocity were analysed. Comparisons between active and inactive period were performed as well as comparisons between groups in the experiments of Chapter 6.

3.1.7. Tissue and blood collection

Mice were euthanized 4 weeks/ 8 weeks after lentiviral vectors injection by sodium pentobarbital overdose. Animals from each group on the C57BL/6J mice study, were randomly selected either for whole brain removal for immunohistochemistry experiments, or for collection of blood, hypothalamic tissue for protein extraction and peripheral organs extraction for histological analysis. Blood was collected upon decapitation and serum was obtained by centrifugation (2,000 × g for 15 min). Serum samples were kept at -20 °C until use. After decapitation, brain was removed and the hypothalamus dissected and stored at -80 °C for posterior processing. After removal of the brain, organs such as the liver, epididimal white adipose tissue (WAT) and intrascapular brown adipose tissue (BAT) were collected and weighed. These organs were then cut and divided, a part of each organ was kept at -80 °C for protein and RNA extraction purposes, while the other was kept in a 10 % neutral buffered formalin solution (VWR International, PA, USA) for 48 h in order to prepare them for histological processing.

For immunohistochemistry, after pentobarbital overdose animals were transcardially perfused with 4 % paraformaldehyde/ phosphate buffer saline (PBS - 137 mM NaCl; 2.7 mM KCl; 10 mM Na2PO4; 1.8 mM KH2PO4; pH 7.4) fixative solution followed by brain removal. Brains were cryoprotected by incubation in a 25 % sucrose/0.1 M PBS solution for 48 h at 4 °C. The brain was frozen and sectioned using a cryostat (LEICA CM3050 S, Leica, Wetzlar, Germany) in 25-µm coronal sections. Slices were collected and stored in 48-well trays, free-floating in 0.1M PBS supplemented with 0.12 µmol/l sodium azide. The plates were stored at 4 °C until immunohistochemical processing.

3.1.8. Biochemical parameters assessment

Glucose levels were measured by using FreeStyle Precision Neo glucometer (Abbott). Cholesterol and triglycerides were measured on Cobas 6000 from Roche (Basel, Switzerland). Serum insulin levels were measure by ELISA kit (Merck Millipore, MA, USA) following manufacturer's instructions.

3.2. Gene expression analysis

3.2.1. Purification and quantification of total RNA

Total RNA extraction for hypothalamus was performed with miRCURY™ RNA Isolation (Exiqon, Copenhagen, Denmark) according to the manufacturer's instructions. Total RNA extraction for liver, WAT and BAT was performed with RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA concentration and purity was determined using a ND-1000 Nanodrop Spectrophotometer (Thermo Fisher Scientific, MA, USA).

3.2.2. Reverse transcription

Reverse transcription into cDNA was carried out by using the iScript Select cDNA Synthesis Kit (Bio-Rad, CA, USA) following the manufacturer's instructions. cDNA samples were then stored at -20 °C until use.

3.2.3. Polymerase chain-reaction

PCR was performed using a specific primer for ataxin-2. The primer for the target gene (mouse Ataxin-2 NM_009125) was pre-designed and validated by QIAGEN (QuantiTect Primers). PCR

reactions were performed in a 25 µL reaction containing 1x PCR Buffer, 2 mM MgCl2, 0.2 mM dNTPs, 2.5 U of DFS-(DNA Free Sensitive) Taq polymerase (Bioron, Ludwigshafen, Germany), 250 nM of each gene specific primer and 1 µL of template cDNA. The reactions were performed in the following sequence of steps: 95 °C for 10 min, followed by 35 cycles at 95 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, and a final extension at 72 °C for 10 min. PCR products were separated by electrophoresis on 2 % agarose gels.

3.2.4. Quantitative real-time polymerase chain reaction

Quantitative Real Time PCR (qRT-PCR) was performed in an iQ5 thermocycler (Bio-Rad) by using 96-well microtiter plates and the IQ SYBR Green Supermix (BioRad). Primers for the target genes were predesigned and validated by QIAGEN and Sigma (Sigma-Aldrich) (Table 3.1). A master mix was prepared for each primer set, containing the appropriate volume of 2× QuantiTect SYBR Green PCR Master Mix and 10× the respective primer. For each reaction, 18 µL of master mix were added to 2 µL of template cDNA. The reactions were performed according to the manufacturer's recommendations. The melting curve protocol started immediately after amplification. The amplification efficiency for each gene and the threshold values for threshold cycle determination (Ct) were determined automatically by the iQ5 Optical System Software (Bio-Rad). Relative mRNA quantification was performed by using the ΔCt method for genes with the same amplification efficiency. The results are expressed as the relative amount compared with control.

Table 3. Primers used for gene expression analysis

Primer	*Temperature*	*Concentration*	*Sequence*	*Function*	*Source*
NPY	55 °C	10 µM	Not provided	Orexigenic neuropeptide	Qiagen
AgRP	58 °C	5 µM	F: AGGTCTAAGTCTGAATGGC R:CGGTTCTGTGGATCTAGC	Orexigenic neuropeptide	Sigma
POMC	58 °C	5 µM	F:AAAAGAGGTTAAGAGCAGTG R:ACATCTATGGAGGTCTGAAG	Anorexigenic neuropeptide	Sigma
TNFα	55 °C	10 µM	Not provided	Pro-inflammatory marker	Qiagen
IL-1β	55 °C	10 µM	Not provided	Pro-inflammatory marker	Qiagen
SOCS3	59 °C	3 µM	F:CCAAAGAAATAACCACTCCC R:GATCTGCGAGGTTTCATTAG	Pro-inflammatory marker	Sigma
TGFβ1	60 °C	5 µM	F:GGATACCAACTATTGCTTCAG R:TGTCCAGGCTCCAAATATAG	Pro-inflammatory marker	Sigma
GFAP	58 °C	5 µM	F:GGAAGATCTATGAGGAGGAAG R:CTGCAAACTTAGACCGATAC	Gliosis marker	Sigma
Hprt	55 °C	10 µM	Not provided	Reference gene	Qiagen
Bmal1	60 °C	3 µM	F:AAATCCACAGGATAAGAGGG R:ATAGTCCAGTGGAAGGAATG	Clock gene	Sigma
Clock	58 °C	3 µM	F:AAGTGACTCATTAACCCCT R:CTATGTGTGCGTTGTATAGTTC	Clock gene	Sigma
Cry	59 °C	3 µM	F:AGAAGGGATGAAGGTCTTTG R:CTCTTAGGACAGGTAAATAACG	Clock gene	Sigma
Per1	58 °C	3 µM	F:GTTCTCATAGTTCCTCTTCTG R:GTGAGTTTGTACTCTTGCTG	Clock gene	Sigma
Per2	55 °C	5 µM	F:CTTTCACTGTAAGAAGGACG R:CTGAGTGAAAGAATCTAAGCC	Clock gene	Sigma
InsR	58 °C	3 µM	F:AAGACCTTGGTTACCTTCTC R:GGATTAGTGGCATCTGTTTG	Insulin receptor	Sigma
IRS1	57 °C	5 µM	F: GATCGTCAATAGCGTAACTG R: ATCGTACCATCTACTGAAGAG	Insulin receptor substrate 1	Sigma

F: Forward primer; R: Reverse primer

3.3. Tissue analysis - Brain

3.3.1. Immunohistochemistry

Hypothalamic ataxin-2 expression was assessed in brain coronal sections of 25 µm. Brain sections were blocked and permeabilized for one hour at room temperature PBS with 10 % (vol/vol) goat serum (Merck Millipore) and 0.3 % Triton X-100 (Merck Millipore). Brain slices were then incubated with mouse monoclonal anti-ataxin-2 antibody (clone 22, 1:2000; BD Biosciences, CA, USA) in blocking solution, overnight at 4 °C. Sections were then incubated with goat anti-mouse Alexa Fluor 594-conjugated secondary antibody (1:200; Invitrogen, CA, USA) for 2 h at room temperature. The nuclei were stained with Hoechst 33342 (2 µg/mL;

Invitrogen). After incubation, brain sections were washed, mounted in slides with Mowiol® mounting medium (Sigma-Aldrich) and analysed on a Zeiss Axiovert fluorescence microscope (Zeiss, Oberkochen, Germany).

3.3.2. Protein lysates of hypothalamus

For the protein extraction protocol, tissue was lysed in 150 µL of radio-immunoprecipitation assay-buffer (RIPA) solution (50 mM Tris-HCl, pH 7.4, 150 nM NaCl (Thermo Fisher Scientific), 1 % Triton X-100; 0.5 % deoxycholate (Sigma-Aldrich); 0.1 % sodium dodecyl sulphate (SDS, Sigma-Aldrich); 200 µM phenyl- methylsulphonylfluoride (PMSF, Sigma-Aldrich); 1 mM dithiothreitol (DTT, Nzytech, Lisbon, Portugal), 1 mM sodium orthovanadate (Na_3VO_4, Sigma-Aldrich); 10 mM sodium fluoride (NaF, Sigma-Aldrich), supplemented with proteases inhibitors (Roche). Tissue was homogenized with a plastic potter and lysates were submitted to a 4-s ultra-sound pulse (1 pulse/s). Lysates were then incubated for 15 min at 4 °C and stored at -20 °C until use.

3.4. Histological analysis of Liver, WAT and BAT

3.4.1. Tissue preparation for Histological analysis

Tissues were collected and placed directly in the 10 % neutral buffered formalin solution (VWR), then submitted to several steps for paraffin (Merck Millipore) blocks inclusion: 1 h at ethanol 70 %; two series of ethanol 95 %, 40 min each; two series of ethanol 100%, 1 h each; two series of xylene (Thermo Fisher Scientific), 1 h each and two series of paraffin, 1 h each. At the end of this process, tissue samples were included in paraffin blocks. Paraffin blocks were sectioned using a microtome (HM325, Thermo Fisher Scientific). The 4 µm thickness sections were placed into microscopy slides until use.

3.4.2. Hematoxilin-Eosin staining

Hematoxylin-eosin staining was performed according to the manufacturer's guidelines (Merck Millipore). Briefly, paraffin sections were deparaffinized in xylene and rehydrated in 100 % ethanol followed by 95 % ethanol. Slides were incubated with Hematoxylin Solution modified acc. to Gill III and then washed in distilled water. Sections were then counterstained with Eosin Y-solution 0.5 % aqueous, washed in distilled water and finally dehydrated in 95 % ethanol,

100 % ethanol and xylene. After staining, sections were mounted in slides with Richard-Allan Scientific Mounting Medium (HM325, Thermo Fisher Scientific) and analysed on a Zeiss Axio Imager Z2 microscope (Zeiss).

3.4.3. White adipocyte tissue analysis

Adipocytes (80-100 *per* animal) with intact cellular membranes were chosen for determination of the cross-sectional area in hematoxylin-eosin stained sections. Images were analysed with Fiji Software (National Health Institute, Bethesda, MD, USA).

3.5. Cellular models

3.5.1. Embryonic Mouse Hypothalamus Cell Line N42 (mHypoE-N42)

The adherent cell line mHypoE-N42 (CELLutions Biosystems Inc./Cederlane, Ontario, Canada) was obtained from immortalized mouse embryonic day 15-18 (E15-18) hypothalamic primary cultures, through retroviral transfer of Simian Vacuolating Virus 40 T- Antigen (SV40 TAg). The cell doubling time is approximately 24 h.

Cells were maintained in high glucose Dulbecco's Modified Eagle Medium (DMEM; 4.5 g/L/ D-glucose, Sigma-Aldrich), supplemented with 10 % heat inactivated fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin (all from Invitrogen, Carlsbad, CA, USA) at 37°C in a humidified atmosphere with 95 % air and 5 % CO_2, in 75 cm^2 tissue culture flasks. When cells reached approximately 90 % confluence, they were washed twice with pre-warmed PBS and detached from the flask by trypsinization, for 3-5 min, at 37 °C. The trypsin (Life Technologies, from Invitrogen) was then inhibited by adding growth medium. Cells were sedimented by centrifugation, at 900 rotations *per* minute (rpm), for 5 min, after which the supernatant was discarded and cells ressuspended in pre-warmed fresh growth medium. Cell density was determined with a hemocytometer, by direct counting and cells were seeded in a new tissue culture flask, for passaging. As for plating, for protein extraction purposes, cells were plated and grown for four days, in uncoated 12-well plates.

Cells were transfected 24 h after plating and 48h after transfection treated either with palmitate or a vehicle solution with bovine serum albumin (BSA).

3.5.2. mHypoE-N42 transfection and palmitate treatment

mHypoN42 cells were plated with a density of 150 000 cells/well in 12-well plates and cultured for 24 h at 37 °C in a 5 % CO_2/air incubator. Cells were then transfected with 1μg of plasmid DNA according with the conditions (Ataxin-2 and ShAtaxin-2) using TorpedoDNA Transfection Reagent (Ibidi, Germany), according to the manufacturer's specifications. Cells were cultured for 24 h at 37 °C in a 5 % CO_2/air incubator before palmitate treatment.

After transfection (24 h), cells were treated with palmitate or a vehicle solution for 24 h. Palmitate solution was obtained by solubilization of 500 μM of palmitic acid (Sigma) and 167 μM of free-fatty-acid BSA (incubation for 2 h at 37 °C), as previously described. A solution of free-fatty-acid BSA was used as control (vehicle). Cells were incubated either with vehicle or palmitate at a final concentration of 500 μM for 24 h. This procedure followed recently published data from Professor Lício Velloso lab (University of Campinas, São Paulo, Brazil), protocol learnt during my stay in this lab inserted in the interchange program Portugal-Brazil FCT-CAPES (*Portovedo et al., 2015*).

3.5.3. Protein lysates of cultured cells

After cell treatments, cell culture plates were immediately placed on ice, the culture media was discarded by aspiration and each well was washed twice with ice-cold PBS. Cells were then lysed with RIPA solution and scrapped of the well. Lysates were incubated for 15 min at 4 °C and stored at -20 °C until use.

3.6. Protein quantification and Western-blotting

Samples (from tissue and cells) were thawed in ice and the insoluble material was pelleted by centrifugation at 16,000 *g* for 10 min at 4 °C. The protein concentration of each sample was determined by the bicinchoninic acid (BCA) protein assay according with manufacturer guidelines (Pierce Biotechnology, IL, USA). Samples were then denaturated, adding sample buffer (0.5 M Tris, 30 % glycerol, 10 % SDS, 0.6 M DTT, 0.012 % bromophenol blue) and boiling for 5 min at 95 °C. Samples were stored at -20 °C until use.

Western Blotting technique was used in order to immunodetect the expression of proteins tissue and cells extracts. The same amount of total protein (60-80 μg) was loaded per lane, separated by electrophoresis in sodium dodecyl sulphate-polyacrylamide (SDS-PAGE) gels (4 %

stacking, 10 % running) and transferred in CAPS buffer (0.1 M CAPS, pH 11.0; 10 % methanol) onto polyvinylidene fluoride (PVDF) membranes (Merck Millipore,). After blotting, membranes were blocked in 5 % non- fat milk in Tris-buffered saline (137 mM NaCl, 20 mM Tris–HCl, pH 7.6) containing 0.1 % Tween 20 (TBS-T) for one hour at room temperature. Immunoblotting was performed overnight at 4 °C using the primary monoclonal antibodies: anti-ataxin-2 (clone 22, 1:1000; BD Biosciences), anti-tubulin (clone SAP.4G5, 1:15000; Sigma), anti-mTOR, anti-phospho-mTOR (Ser2448), anti-RPS6K and antiphospho-RPS6K (Thr389) (1:1000, Cell Signallling, MA, USA). After incubation, membranes were washed three times with TBS-T and incubated with an alkaline phosphatase-linked secondary antibody, specific mouse IgG or rabbit IgG in a 1:10000 dilution (Invitrogen). Protein immunoreactive bands were visualized by chemifluorescence using the ECF (enhanced chemiofluorescence) substrate (GE Healthcare, Little Chalfont, UK) in a VersaDoc Imaging System (Bio-Rad) and the optical density of the bands was quantified with the Quantity One Software (Bio-Rad). The results are normalized to β-tubulin and are expressed as the relative amount compared with control.

3.7. Statistics

Results are expressed as mean ± standard error of the mean (SEM). Data relative to weight gain was analysed using a two-way analysis of variance (ANOVA) to evaluate the effect of time and treatment (hypothalamic modulation of ataxin-2). Data was analyzed using Student's unpaired t test with two-tailed p value. Analysis was performed between the two groups (Control and treatment) of the corresponding diet (Chow or HFD). In Chapter 7, the analysis of animal data was also always performed between two groups, either WT and KO or KO and KO+Ataxin-2. Heterozigous group was used only as a reference for an intermediate expression of ataxin-2 between WT and KO. A value of $p<0.05$ was considered significant. Prism 6.0 (GraphPad Software) was used for all statistical analysis.

CHAPTER 4

Ataxin-2 hypothalamic silencing mimics diet-induced obesity

4.1. Abstract

Obesity is one of the major causes for high morbidity and morbility diseases such as type 2 diabetes and cardiovascular diseases. Fat enriched diets promote an overall pro-inflammatory environment that promotes dysregulation of metabolic homeostasis. The hypothalamus, namely the arcuate nucleus, is susceptible to overall inflammation due to its ability of periphery stimuli sensing. Hypothalamic inflammation mediates the disruption of lipid metabolism, glucose sensing and insulin sensitivity. Finding new players to fight or revert obesity-induced damages in the hypothalamus and other metabolic organs might constitute a therapeutic approach for obesity-related conditions. Ataxin-2 acts in different hypothalamic functions such as body weight regulation, circadian rhythm and stress response. Moreover, others showed that ataxin-2 knockout mice (ATXN2 KO) are obese, have dyslipidaemia and insulin resistance. These observations suggest that ataxin-2 may have a regulatory role on body weight homeostasis. However, the specific role of ataxin-2 in the hypothalamus was never investigated before.

The aim of the present study was to investigate the role of hypothalamic ataxin-2 on energy homeostasis. Our first results showed that mice under HFD that are obese, have lower hypothalamic ataxin-2 levels, compared to mice fed with chow diet.

In order to further investigate the effect of lower levels of ataxin-2 in the hypothalamus, we specifically silenced ataxin-2 in mice arcuate nucleus and evaluated body weight changes, insulin sensitivity, glycaemia, pro-inflammatory markers levels, and structural changes of metabolic organs (liver, white and brown adipose tissue).

The results show that mice with a decrease of ataxin-2 in the arcuate nucleus (around 50 %) are obese, insulin resistant, have hyperglycaemia and higher levels of pro-inflammatory markers (TNF-α, IL-1β, TGFβ1, SOCS3 and GFAP) in the hypothalamus, compared to control mice. Moreover, ataxin-2 silencing in the hypothalamus increased hepatic lipid accumulation, white adipose tissue enlargement and altered brown adipose tissue physiology. The changes observed in peripheral organs upon ataxin-2 silencing in mice fed a chow diet were in fact similar to those observed upon HFD feeding.

Altogether, the results show that ataxin-2 silencing in the hypothalamus in chow diet was enough to mimic some obesity characteristics, such as insulin resistance and hypothalamic inflammation. These study suggests a new role for ataxin-2 in the hypothalamus and suggest that hypothalamic ataxin-2 is a new player to be explored in the context of obesity.

4.2. Introduction

Obesity incidence increased in the last decades. Obesity increases the incidence of type 2 diabetes, cardiovascular diseases, liver disease, neurodegeneration and some cancer types (*Wellen and Hotamisligil, 2005; Hotamisligil, 2006; Brown et al., 2009*). It is well accepted that obesity is characterized by a low-grade and chronic inflammation that is directly associated to the metabolic consequences of increased body weight (*Shoelson et al., 2006; Hotamisligil, 2006; Baker et al., 2011; Odegaard and Chawla, 2013*).

Inflammation can lead to the dysfunction of several organs, including the brain. In the brain, the hypothalamus is the first region to be affected by obesity-induced inflammation (*de Souza et al., 2005; Zhang et al., 2008; Posey et al., 2009; Valdearcos et al., 2015*). The hypothalamus is the key neuroendocrine center, with specialized neurons in distinct nuclei, which via neural pathways and neuroendocrine hormones is able to regulate energetic homeostasis (*Schwartz et al., 2000; Barsh and Schartz, 2002; Morton et al., 2006; Waterson and Horvath, 2015*). The arcuate nucleus of the hypothalamus (ARH) integrates signals from the periphery and coordinates a response according to the overall nutritional status (*Dietrich and Horvath, 2009; Belgardt and Brunning, 2010; Meyers et al., 2010*). This response is achieved through the activity of two neuronal populations within ARH, the AgRP/NPY (Agouti related protein/Neuropeptide Y) neurons that are orexigenic neurons (increases food intake) and the POMC/CART (Pro-opiomelanocortin/Cocaine- and amphetamine-regulated transcript) neurons that are anorexigenic neurons (inhibit food intake). AgRP/NPY neurons receive excitatory inputs from ghrelin to promote food intake, while POMC/CART receive excitatory inputs from insulin and leptin to inhibit food intake and promote energy expenditure (*Sohn, 2015; Waterson and Horvath, 2015*). Inflammation resulting from obesity can impair these nutrient sensing pathways, and change the balance between food intake and energy expenditure, further promoting weight gain (*Cai, 2013; Tang et al., 2015*).

The ARH also participates in the hypothalamic control over periphery metabolic organs such as the liver, white adipose tissue (WAT), brown adipose tissue (BAT), pancreas and skeletal muscle (*Seoane-Collazo et al., 2015*). The hypothalamic inflammation that occurs in obesity induces dysfunctions in those peripheral organs resulting on hyperglycaemia, dyslipidaemia, hypertension and insulin resistance (*Milanski et al., 2008; Arruda et al., 2011; Valdearcos et al., 2015*).

Ataxin-2 is involved in several hypothalamic-regulated functions, namely energy balance (*Kiehl*

et al., 2006; Lastres-Becker et al., 2008b), fertility (*Ciosk et al., 2004; Scoles et al., 2012*), circadian rhythms (*Lim and Allada, 2013; Zhang et al., 2013; Pfeffer et al., 2016*), stress response (*Huynh et al., 2009; Amir-Zilberstein, 2012*) and aging (*Sebastiani et al., 2012; Salvi et al., 2014*). In the last couple of years some studies suggest that ataxin-2 is relevant of metabolic regulation (reviewed in *Carmo-Silva et al., 2017*).

Ataxin-2 gene (*ATXN2*), localized in chromosome 12q24.1, is present in several brain regions, especially in cerebellum (Purkinje cells), but also in the liver and the heart (*Huynh et al., 1999; Fittschen et al., 2015*). Ataxin-2 is involved in several cellular processes, from RNA metabolism to calcium signaling (reviewed in *Magaña et al., 2012*; *Yokoshi et al., 2014; Gnazzo et al., 2016*).

Interestingly, a mutation in ATXN2 gene was correlated with polyphagia and obesity (*Abdel-Aleem and Zaki, 2008*), whereas the knockout of ataxin-2 in mice promoted obesity, insulin resistance and lipid disturbances (*Kiehl et al., 2006; Lastres-Becker et al., 2008b*). Recently, ataxin-2 was showed to be involved in lipid metabolism and in nutrient-sensing metabolic pathways, acting on mTOR and AMPK signaling (reviewed in *Carmo-Silva et al., 2017*). All these observations suggest that ataxin-2 might act as a metabolic modulator.

The aim of this study was to gain insight into the impact of ataxin-2 in the central metabolic regulation, namely the role of ataxin-2 in the hypothalamus. We observed that obesity could decrease hypothalamic ataxin-2 levels, therefore we investigated the specific silencing of ataxin-2 in the ARH. With this purpose, we used lentiviral vectors encoding for a short hairpin targeting ataxin-2 (for silencing) and injected these viral particles in the ARH of C57BL/6J mice. These mice were fed either with a chow diet or with a HFD for four weeks. During the four weeks we assessed body weight, food intake and insulin sensitivity. In the end of the study we evaluated hypothalamic neuropeptides, pro-inflammatory markers in the hypothalamus and structure of metabolic organs (liver, white and brown adipose tissue).

4.3. Results

Ataxin-2 is expressed in metabolic organs, enriched in the arcuate nucleus of the hypothalamus and decreased upon HFD

Previous studies have shown ataxin-2 in present in the brain and in other organs, namely in the hypothalamus and in metabolically active organs, such as the liver (*Huynh et al., 1999; Fittschen et al., 2015*). In line with these observations we also observed that ataxin-2 is present in the hypothalamus of C57Bl/6J mice (Figure 4.1A). Moreover, for the first time, we show that ARH is the hypothalamic nucleus with higher ataxin-2 quantity (Figure 4.1A). Furthermore, ataxin-2 is also present in metabolically organs such as in the liver, white and brown adipose tissue (Figure 4.1B).

Recent evidences show that in mammalian cells, ataxin-2 levels increase upon starvation (*Lastres-Becker et al., 2016*). Moreover, it was also shown that ataxin-2 mediates caloric restriction effects (*Salvi et al., 2014; Bar et al., 2016*). In the present work, when we compared hypothalamus of mice fed with normal chow diet to hypothalamus from mice fed with high fat diet (HFD) for 4 weeks, we observed that hypothalamic ataxin-2 protein levels are significant lower in the hypothalamus from mice under high fat diet, when compared to mice in chow diet (Figure 4.1C). These results suggest that ataxin-2 levels depend on nutritional environment.

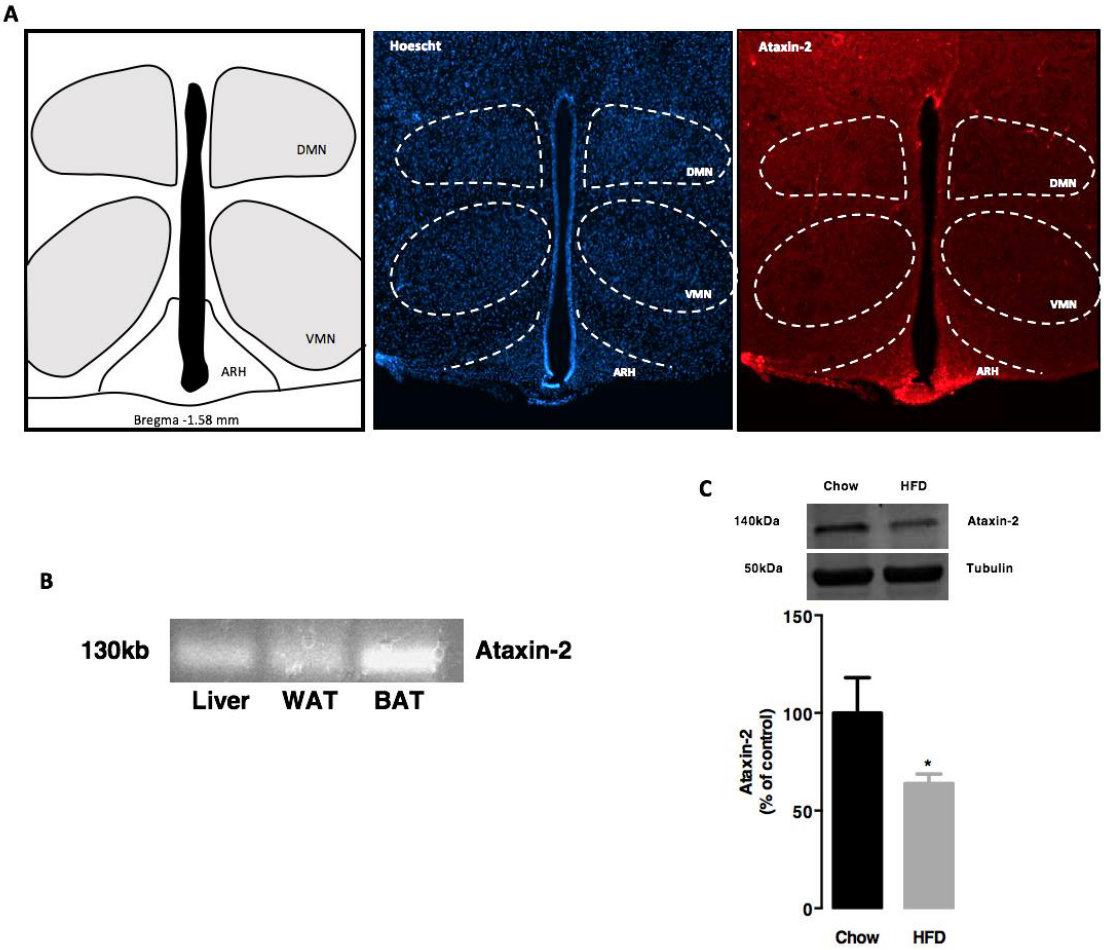

Figure 4.1. Ataxin-2 is present in higher amounts in arcuate nucleus of the hypothalamus (ARH; A) and liver, WAT and BAT (B). HFD induces a decrease on ataxin-2 levels in ARH (C). (A) Schematic representation of hypothalamic nuclei. Representative images of hypothalamic nuclei, stained with nuclear marker (Hoechst - blue) and ataxin-2 (red) staining in the arcuate nucleus of the hypothalamus. Dashed area represents the different nuclei of the hypothalamus. (B) DNA blot analysis of the amplification product of the PCR for ATXN2 in hypothalamus, liver, white adipose tissue (WAT) and brown adipose tissue (BAT). (C) Western blotting analysis of ataxin-2 protein levels in the whole hypothalamus of mice fed with a high fat diet (HFD) or chow diet for 4 weeks. Data is expressed as the mean ± SEM for n=3-5 per condition. *$p < 0.05$ compared to control (chow). ARH: arcuate nucleus of the hypothalamus; VMN: ventromedial nucleus; DMN: dorsomedial nucleus

Lentiviral vectors encoding for a short-hairpin against ataxin-2 efficiently decreased ataxin-2 levels in the hypothalamus

In order to evaluate the effect of ataxin-2 in the hypothalamus, ataxin-2 was silenced in the ARH, by gene transfer using lentiviral vectors, in male C57BL/6J mice. Mice were injected with lentivirus encoding either GFP (Control group) or a short-hairpin targeting ataxin-2 for promoting ataxin-2 silencing (ShAtx2 group), under a neuronal-specific promoter by bilateral

stereotaxic injection in each ARH (*Aveleira and Botelho et al., 2015*). After 4 weeks, brains were isolated and processed for immunohistochemistry and RNA extraction, to analyse the expression of ataxin-2 in the hypothalamus. In control mice brains, as observed previous (Figure 4.1A), ataxin-2 immunoreactivity was mostly restricted to the ARH. Mice injected with ShAtaxin-2 have lower levels of ataxin-2 in the ARH (63.4 ± 2.4 % of control, Figure 4.2B) in comparison to control mice (Figure 4.2A, B). In accordance, ShAtaxin-2 mice have lower levels of ataxin-2 mRNA, compared to control mice, (ShAtaxin-2; 52.5 ± 6.2 % of control, Figure 4.2C).

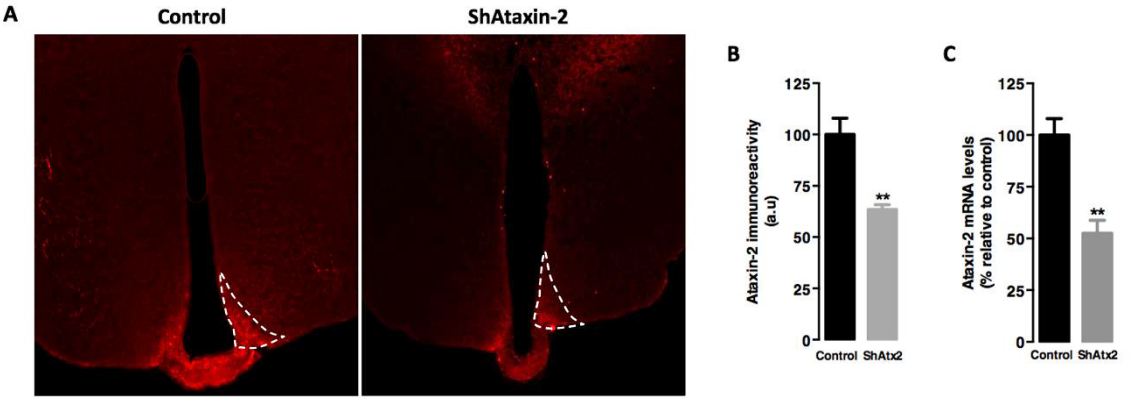

Figure 4.2. Injection of lentiviral vector ShAtaxin-2 in the ARH decreased ataxin-2 immunoreactivity in ARH (A, B) and ataxin-2 mRNA levels in the whole-hypothalamus (C). (A-B) ShAtaxin-2 decreased ataxin-2 in the ARH. (A) Representative images of ataxin-2 immunoreactivity in the ARH of the hypothalamus. Dashed area represents the arcuate nucleus of the hypothalamus. (B) Quantification of ataxin-2 immunoreactivity through the anterior-posterior length of the mouse ARH, four weeks after lentiviral injection. Control chow n= 3; ShAtaxin-2 chow n=3. The results represent the mean ± SEM and are expressed as the relative amount compared to Control group. ***p*<0.01, compared to control. (C) Lentiviral delivery of a short-hairpin targeting ataxin-2, decreased ataxin-2 mRNA expression in the whole-hypothalamus. Ataxin-2 mRNA content relative to control. Mice were fed with chow diet. Control n=3-5; ShAtaxin-2 n=5. Data is expressed as the mean ± SEM. ***p*<0.01, compared to Control.

Specific silencing ataxin-2 in ARH increases food intake and body weight gain

Since we observed that HFD decreased ataxin-2 levels in the hypothalamus, we investigated if a specific decrease of ataxin-2 in ARH could change energy balance regulation. For this purpose, we silenced ataxin-2 expression in the ARH using lentiviral vectors encoding for a short-hairpin for ataxin-2 silencing (ShAtaxin-2). As controls, we used mice injected with lentiviral vectors encoding GFP in ARH. Each one of these groups (GFP or ShAtaxin-2 injected) was divided in two subgroups, one fed with chow diet and the other with HFD for 4 weeks.

Hypothalamic ataxin-2 silencing induced a trend to body weight increase, in both chow and HFD groups (Figure 4.3A, B). Although this weight gain was only statistically significant different from controls in mice fed with chow diet. The non-significant results in the HFD group might be result of high heterogeneity resulting from a distinct individual response to the diet or due to different basal expression of ataxin-2, since HFD *per se* decreases ataxin-2 levels (Figure 4.1C). However, mice in chow and HFD with ataxin-2 hypothalamic silenced, eat more (hyperphagia) when compared to controls (Figure 4.3C).

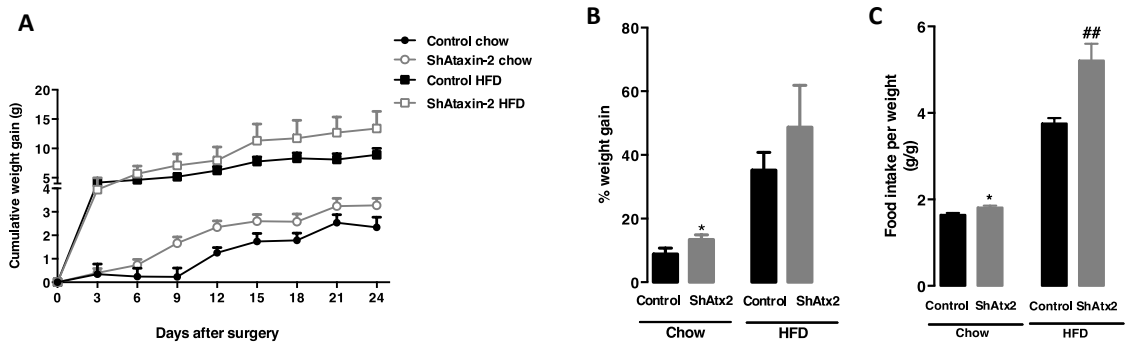

Figure 4.3. Ataxin-2 silencing in the ARH increases food intake and body weight gain. Control mice were injected with lentivirus encoding for GFP and the ShAtx2 groups were injected with a shorthairpin for ataxin-2 silencing (ShAtaxin-2). Mice were fed with chow diet and high fat diet (HFD) for 4 weeks, after injection. (A, B) Ataxin-2 silencing in the hypothalamus promotes increased weight gain. Weight gain represented as (A) cumulative weight gain (g) and as (B) percentage of weight gain between the beginning and end of the study. (C) Ataxin-2 silencing in the hypothalamus promotes hyperphagia. Total food intake (4 weeks) expressed in a ratio with animal weight (g/g). Control chow n= 6; ShAtaxin-2 chow n=8; Control HFD n=9; ShAtaxin-2 HFD n=6. Data is expressed as the mean ±S EM. **p*<0.05 compared to Control chow $^{\#\#}p$<0.01 compared to Control HFD.

Silencing hypothalamic Ataxin-2 induces hyperglycaemia and insulin resistance

ATXN2 KO mice develop insulin resistance (*Lastres-Becker et al., 2008b*), which might be a consequence of obesity or changes on insulin signalling. By measuring glycaemia and insulin tolerance test (ITT), we investigated whether ataxin-2 ARH silencing affects insulin sensitivity. Interestingly, under chow diet, mice with ataxin-2 silencing have higher fasting glucose levels compared to controls (Control 141.6 ± 21.5 *versus* ShAtx2 209.1 ± 6.1, Figure 4.4A). Under HFD, no difference on fasting glucose levels between controls or ataxin-2 silencing mice were observed (Figure 4.4A). Accordingly, only in chow group, the hypothalamic ataxin-2 silencing impaired blood glucose clearance by insulin, as observed in the ITT (Figure 4.4B) and calculated

by the kITT (glucose clearance rate) (Figure 4.4C). These results suggest that ataxin-2 in the hypothalamus can be a mediator of glucose metabolism regulation.

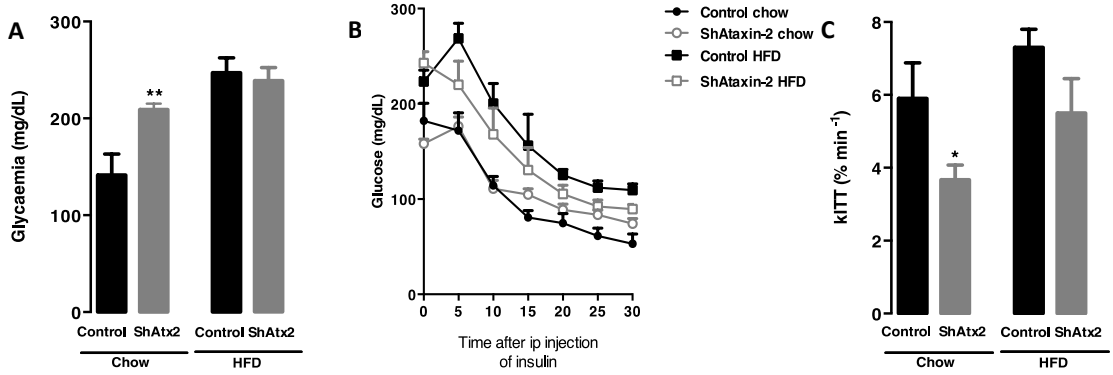

Figure 4.4. Mice with lower levels of ataxin-2 in the ARH in chow fed mice are hyperglycaemic and insulin resistance. (A) Ataxin-2 silencing in ARH increases glycaemia in chow fed mice. Glycaemia (mg/mL) in Control and ShAtx2 mice fed with chow diet and high fat diet. (B) Hypothalamic ataxin-2 silencing impairs insulin sensitivity. Insulin tolerance test (ITT) of Control and ShAtx2 mice fed with chow and high fat diet, expressed as the glycaemia (mg/mL) measured every 5 minutes after intraperitoneal injection of insulin. (C) Ataxin-2 silencing impairs glucose clearance by insulin. kITT representing the glucose clearance rate (% per minute) in chow and high fat diet for the Control and ShAtx2 groups. Control chow n= 7-9; ShAtaxin-2 chow n=8; Control HFD n=3; ShAtaxin-2 HFD n=3. Data is expressed as the mean ± SEM. *p<0.05 and **p<0.01, compared to Control chow.

Ataxin-2 silencing in ARH changes hepatic lipid content

Obesity and insulin resistance are associated with peripheral organs alterations, namely with liver steatosis (*Shimizu et al., 2014; Shimizu and Walsh, 2015; Xiaona et al., 2016*). Hematoxilin and Eosin (H&E) staining was performed on liver samples of the groups (Figure 4.5A) in order to access the effect of hypothalamic ataxin-2 modulation in these peripheral alterations.

According to recent NASH scoring (*Liang et al., 2014*), liver steatosis can be accessed by the presence and scoring of certain histological findings such as: macrovesicular steatosis (large lipid droplets in hepatocytes), microvesicular steatosis (small lipid droplets hepatocytes) and hypertrophy of hepatocytes. In our study we observed that hypothalamic silencing of ataxin-2 led to hypertrophy of hepatocytes and microvesicular steatosis (Figure 4.5A). Under HFD, mice were obese and presented liver lipid accumulation (Figure 4.5A). In HFD, ataxin-2 silencing worsened liver pathology (Figure 4.5A). Lipid disturbances upon ataxin-2 silencing can also be observed in the serum with the increase in triglycerides (Control 86 ± 13.2; ShAtaxin-2 125 ± 13.8, Figure 4.5B) and in cholesterol (Control 75.8 ± 3.2; ShAtaxin-2 92.3 ± 3.7, Figure 4.5C)

levels in chow diet group. Overall, the hypothalamic ataxin-2 silencing in chow diet promoted changes typical of an obesity context.

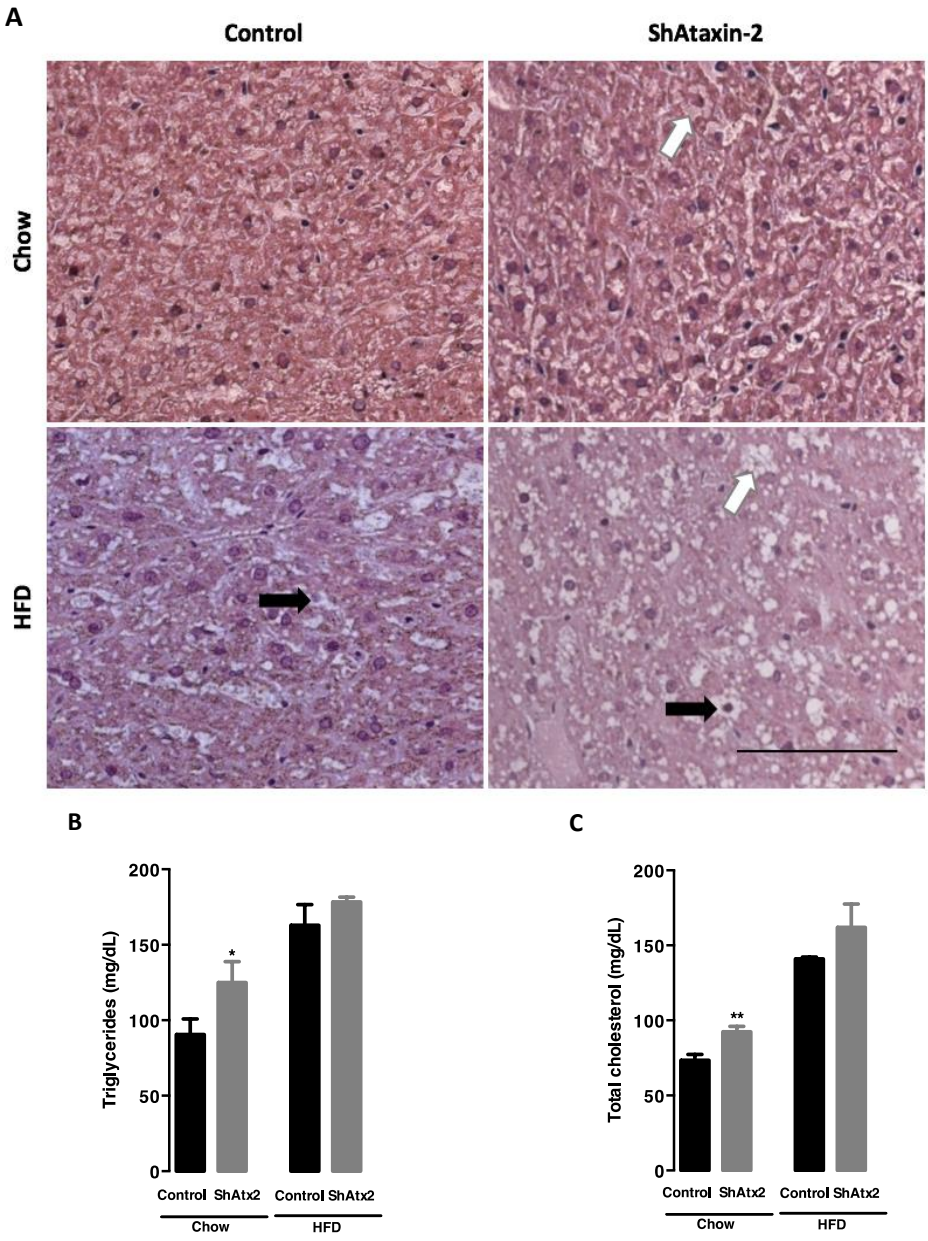

Figure 4.5. Silencing ataxin-2 in ARH of mice under chow diet increases lipid accumulation in liver, increases serum triglycerides and cholesterol levels (A) Ataxin-2 silencing in the ARH promotes lipid accumulation and hypertrophy of hepatocytes. H&E stained sections from livers of mice fed either chow or HFD for 4 weeks, in ataxin-2 silencing groups. Microvesicular steatosis (black arrow) and hypertrophy (white arrow). For each group n=3-4, scale bar 100 µm. (B) Hypothalamic ataxin-2 silencing promotes an increase in triglycerides in mice on chow diet. Triglycerides (mg/mL) content in serum of mice fed with chow and high fat. (C) Hypothalamic ataxin-2 silencing increases total cholesterol in chow fed mice. Total cholesterol (mg/mL) in the serum of mice fed with chow and high fat diet in the Control and ShAtx2 groups. Data is expressed as the mean ± SEM. Control chow n= 6; ShAtaxin-2 chow n=8; Control HFD n=9; ShAtaxin-2 HFD n=6. *p<0.05 and **p<0.01 compared to Control chow.

Hypothalamic ataxin-2 silencing increases white adipocytes diameter

WAT enlargement is an obesity feature, where adipocytes increase in size in order to contain the lipid excess present in this condition. Adipocyte size, fibrosis and infiltration of immune cells are characteristics usually analysed in the study of WAT (*Sun et al., 2012; Xiaona et al., 2016*). WAT samples from the 4 groups of mice were stained with H&E to evaluate adipocyte size (Figure 4.6A). Although the qualitative analysis of the images suggests that hypothalamic ataxin-2 silencing (ShAtaxin-2) increases adipocyte size in chow fed mice (Figure 3.6A), animal variability does not allow for statistic differences in adipocyte area between control and ShAtaxin-2 groups both in chow and HFD (Figure 3.6B).

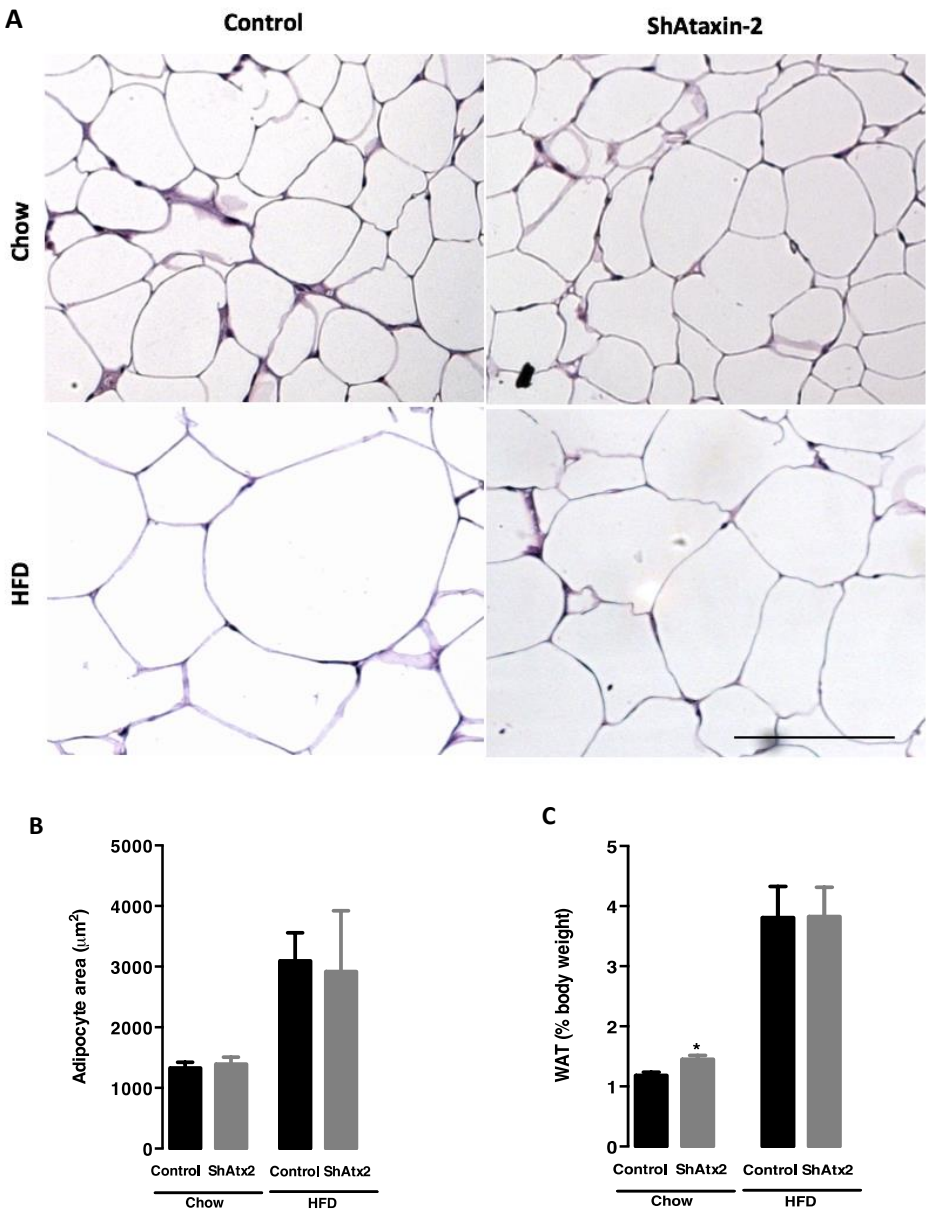

Figure 4.6. Hypothalamic ataxin-2 silencing promotes white adipocyte enlargement. (A) Representative images of H&E staining of WAT of mice fed either chow or HFD for 4 weeks. For each group n=3-4, scale bar 100 µm. (B) Adipocyte diameter upon chow diet and HFD in control and ataxin-2 silencing groups. (C) Ataxin-2 silencing in the ARH promotes an increase in WAT in mice fed with chow diet. Epididymal WAT weight % relative to body weight. Data is expressed as the mean ± SEM. Control chow n= 6; ShAtaxin-2 chow n=8; Control HFD n=9; ShAtaxin-2 HFD n=6. **p*<0.05 compared to Control chow.

Consistent with the increased weight gain observed upon ataxin-2 silencing in chow diet, we observed an increase in the WAT % in the ShAtx2 group compared to control (Figure 4.6C). As expected, HFD increased WAT % relative to total body weight, in control and ShAtx2 mice. (Figure 4.6C). In the HFD groups there is no difference WAT weight % between controls and ShAtx2 mice (Figure 4.6C).

Hypothalamic ataxin-2 silencing induced BAT whitening

BAT "whitening" can be a consequence of obesity, resulting from the loss of vascularity and hypoxia in this organ (*Shimizu et al., 2014; Shimizu and Walsh, 2015*). H&E staining of BAT from control mice showed small, multilocular adipocytes (Figure 4.7A), whereas from mice under HFD, the adipocyte size lipid droplet increased resembling white adipocytes (Figure 4.7A). Mice under chow diet with ataxin-2 hypothalamic silencing have increased number of large unilocular adipocytes in BAT (Figure 4.7A). In HFD, ataxin-2 silencing also changes BAT, compared to control, with bigger adipocytes and the presence of several smaller lipid droplets. Overall these results suggest that hypothalamic ataxin-2 has a role in BAT physiology and its decrease in the hypothalamus can promote changes in BAT, similar as observed in obesity.

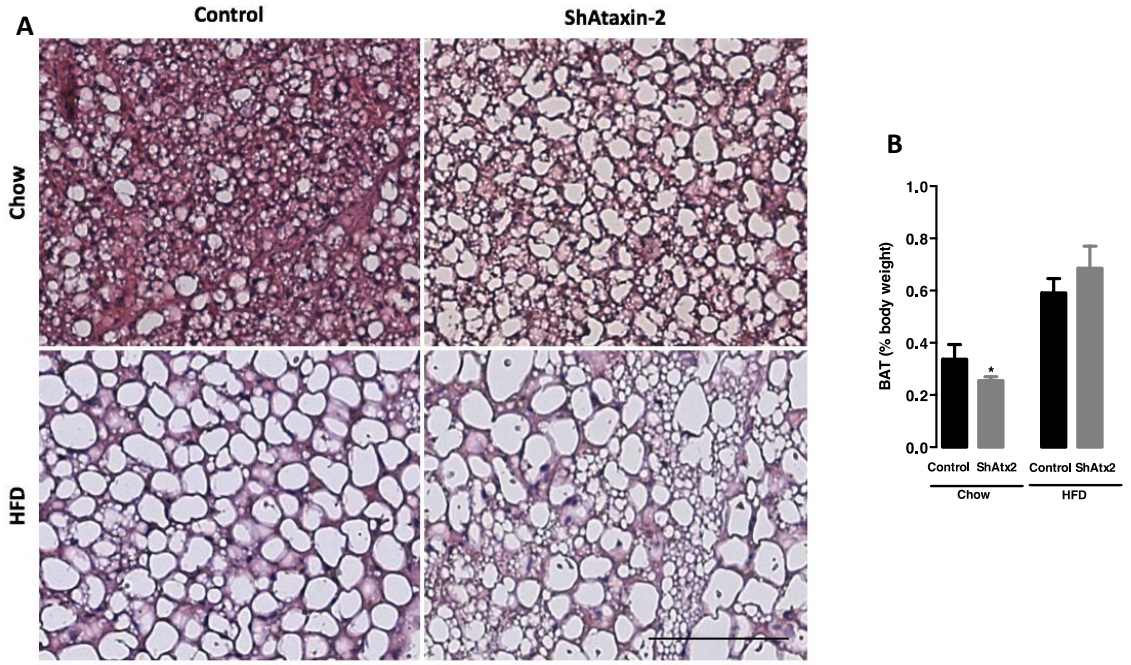

Figure 4.7. Hypothalamic ataxin-2 silencing decreases BAT weight and increases BAT "whitening". (A) Representative images of H&E stained sections from interscapular brown adipose tissue (BAT) of mice fed either chow or HFD for 4 weeks. For each group n=3-4, scale bar 100 µm. (B) Ataxin-2 silencing in the ARH promotes a decrease in mice fed with chow diet. BAT weight % relative to body weight. Data is expressed as the mean ± SEM. Control chow n= 6; ShAtaxin-2 chow n=8; Control HFD n=9; ShAtaxin-2 HFD n=6. **p*<0.05 compared to Control chow.

Ataxin-2 silencing changes food intake neuropeptides expression: increases orexigenic peptides (NPY and AgRP) but decreases anorexigenic peptide POMC

The hypothalamic regulation of food intake and energy expenditure relies on the balance between orexigenic and anorexigenic neuropeptides. Orexigenic neuropeptides, such as NPY and AgRP, increase food intake whereas anorexigenic neuropeptides, such as POMC, decrease food intake and promote energy expenditure (*Schwartz et al., 2000; Morton et al., 2014; Waterson and Horvath, 2015*). In obesity there is a compromise of this balance and the dysregulation in the normal levels of neuropeptides (*Bergen et al., 1999; Levin et al., 1997; Ziotopoulou et al., 2000; Gao et al., 2002*). Hypothalamus from mice under chow diet and with ataxin-2 hypothalamic silencing have higher levels of NPY (375.9 $\pm$ 89 % of control; Figure 4.8A) and AgRP (385.2 $\pm$ 113.4 % of control; Figure 4.8B) mRNA content, but lower levels of mRNA of the anorexigenic neuropeptide POMC (42.5 $\pm$ 18.6 % of control; Figure 4.8C). Hypothalamic silencing of ataxin-2 in mice fed with HFD also increased AgRP (303 $\pm$ 149 % of control HFD) and decreased POMC (28.1 $\pm$ 8.2 % of control HFD). Ataxin-2 silencing did not change NPY mRNA content (109 $\pm$ 27.8 % of control HFD). Although these results are concordant to those observed in chow diet, as observed previously, HFD decreased ataxin-2 levels (Figure 4.1C). For this reason, the results observed might not translate correctly the impact of ataxin-2 silencing in the neuropeptide expression, but a cumulative effect of the diet and the silencing. However, these observations are consistent with the increased food intake presented by the ShAtaxin-2 group fed with HFD.

The dysregulation of orexigenic/anorexigenic neuropeptides balance favouring the orexigenic input might be the cause of hyperphagia observed in mice with ataxin-2 hypothalamic silencing.

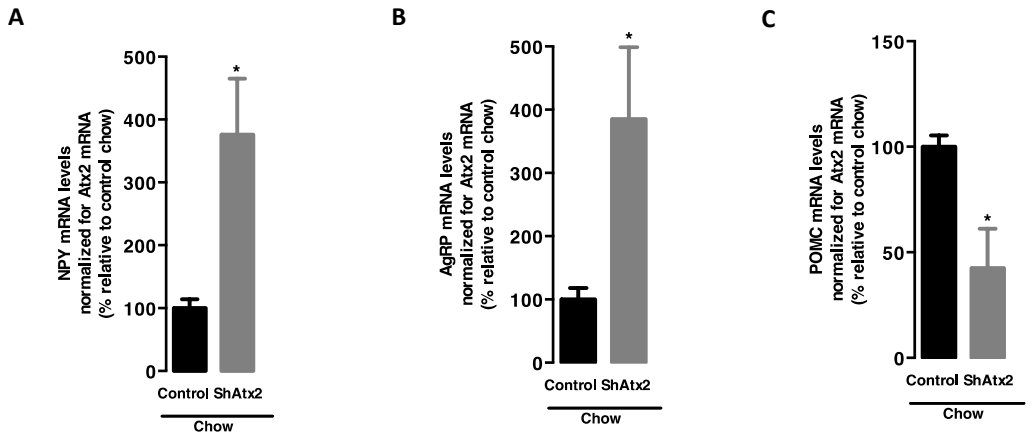

Figure 4.8. Ataxin-2 silencing in the ARH increases orexigenic neuropeptides (AgRP and NPY) but decreases anorexigenic neuropeptide (POMC)**.** The mRNA levels of NPY (A) AgRP (B) and POMC (C) were evaluated in hypothalamus with ARH ataxin-2 silencing and fed with chow diet; mRNA levels normalized to Atx2 mRNA content to overcome transduction differences. Control chow n= 3-5; ShAtaxin-2 chow n=5. Data is expressed as the mean ± SEM. **p*<0.05, compared to control.
AgRP: Agouti related protein; NPY: Neuropeptide Y; POMC- Pro-opiomelanocortin

ARH ataxin-2 silencing contributes to a pro-inflammatory environment in the hypothalamus

Hypothalamic inflammation is one of the major causes of metabolic malfunctioning and an important consequence of obesity (*Velloso and Schwartz et al., 2011; Williams, 2012; Cai, 2013; Valdearcos, 2015*). Obesity and HFD consumption *per se* promote several changes in the hypothalamus such as the increase in pro-inflammatory cytokines, astrogliosis and deregulation in leptin and insulin signaling (*Milansky et al., 2009; Thaler et al., 2012; Horvath et al., 2010; Yan et al., 2014*). Hypothalamus from mice with ataxin-2 hypothalamic silencing under chow diet presented an increase of pro-inflammatory factors levels. So, ataxin-2 silencing *per se* increased TNF-α (629 $\pm$ 167.9 % of control, Figure 4.9A), IL-1β (398.3 $\pm$ 72.1 % of control, Figure 4.9B), TGFβ1 (749.5 $\pm$ 110.3 % of control, Figure 4.9C), SOCS3 (474.1 $\pm$ 87.9 % of control, , Figure 4.9D) and GFAP (291 $\pm$ 68.4 % of control, Figure 4.9E). Overall, the silencing of hypothalamic ataxin-2 in mice under chow diet seems to mimic the hypothalamic inflammation caused by HFD consumption and obesity

The silencing ataxin-2 in the ARH of mice fed with HFD, compared to control HFD, did not significantly change the pro-inflammatory markers (TNF-α: 144 $\pm$ 59 % of control HFD; TGF1β: 142 $\pm$ 39.7 % of control HFD; SOCS3: 156 $\pm$ 73.3 % of control).

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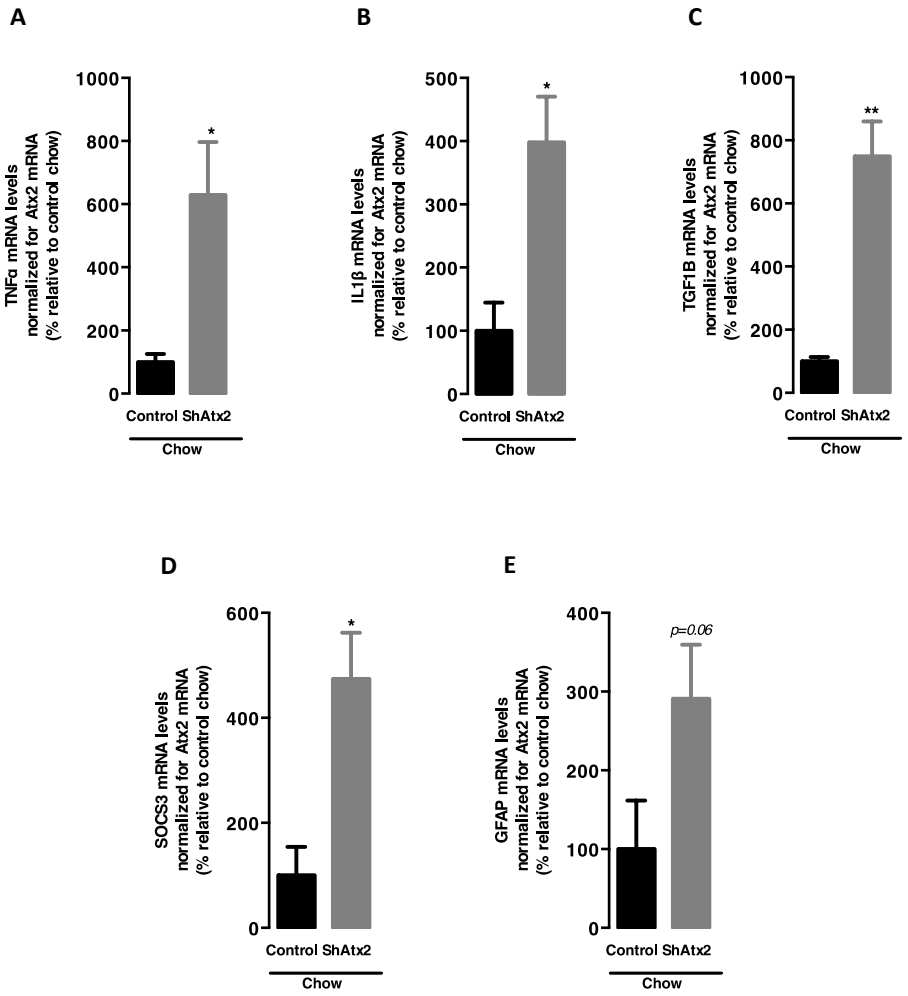

Figure 4.9. Ataxin-2 silencing in the ARH in chow-fed mice increases pro-inflammatory markers in the hypothalamus. Hypothalamic mRNA levels of inflammatory markers in mice fed with chow diet, control versus mice with hypothalamic ARH ataxin-2 silencing. (A) TNF-α, (B) IL-1β, (C) TGF1β, (D) SOCS3 and (E) GFAP mRNA content relative to control. Control chow n= 3-5; ShAtaxin-2 chow n=5. Data is expressed as the mean ± SEM. *p<0.05 and **p<0.01, compared to control.

GFAP: Glial fibrillary acidic; IL-1β: Interleukine 1 beta; TGFβ-1: Transforming growth factor beta 1; TNF-α: Tumor necrosis factor alpha; SOCS3: Supressor of cytokine signaling 3.

4.4. Discussion

Improving the understanding of the networks that regulate energy balance, specially finding new interactive players that mediate these processes, is the key to better understand obesity and related disorders. In the present study we specifically silenced ataxin-2 in the ARH which resulted on food intake and body weight gain increase, insulin resistance and modifications in peripheral metabolic organs, such as liver, WAT and BAT. These results suggest that hypothalamic ataxin-2 plays a role in energy balance regulation and systemic metabolism.

Here we show that ataxin-2 is expressed in the mouse hypothalamus, with a significant enrichment in the ARH, a key region that integrates peripheral signals from nutrients and hormones in order to regulate food intake, energy expenditure and glucose metabolism (*Waterson and Horvath, 2015; Seoane-Collazo et al., 2015; Lage et al., 2016).*

We observed for the first time that 4 weeks of HFD decreases ataxin-2 levels in the hypothalamus. Others showed that starvation promotes *ATXN2* expression in human SH-SY5Y neuroblastoma cells (*Lastres-Becker et al., 2016*). These findings suggest ataxin-2 levels are modulated by the nutritional status.

Given the obese phenotype of the ATXN2 KO mice (*Kiehl et al., 2006; Lastres-Becker et al., 2008b*), and ataxin-2 as a metabolic regulator (reviewed in *Carmo-Silva et al., 2017*), perhaps the absence of this protein in the hypothalamus is behind the observed weight gain. In order to study ataxin-2 involvement in hypothalamic functions, we silenced ataxin-2 specifically in the ARH in chow and HFD fed mice for 4 weeks. We observed that hypothalamic ataxin-2 silencing in chow fed mice promoted an increase in food intake, accompanied by increased body weight gain, mimicking the effects of a HFD. However, in mice under HFD, ataxin-2 silencing in the hypothalamus increased food intake but did not further increase body weight, when compared to control mice under HFD. This phenomenon might arise from the already severely compromised metabolic status caused by the HFD.

To further investigate the mechanisms underlying the food intake increase induced by silencing ataxin-2 in the ARH, we evaluated the levels of neuropeptides that regulate food intake (*Barsh and Schwartz, 2002; Begg and Woods, 2013; Morton et al., 2014*). ARH ataxin-2 silencing, in mice under chow diet, increased orexigenic neuropeptides mRNA levels, AgRP and NPY, and decreased the anorexigenic POMC. Ataxin-2 silencing in mice under HFD promoted a similar trend to that observed in chow, however changes in hypothalamic NPY, AgRP nor POMC levels were not significantly different when compared to mice in HFD. The hypothalamic neuropeptide modulation upon HFD is still a controversial subject, some studies report an

increase in orexigenic inputs and a decrease in anorexigenic signalling, while others report the exact opposite (*Gao et al., 2002; Torri et al., 2002; Briggs et al., 2010*). Therefore, in the context of HFD is it hard to actually discern the effect of ataxin-2 silencing, given the heterogeneity of individual responses of mice upon different types of diets. However, it is widely accepted that an ineffective compensatory mechanism between POMC and NPY/AgRP can be responsible for obesity predisposition. Our results suggest that ataxin-2 silencing changes the hypothalamic neuropeptides towards an orexigenic stimulus, which may account for the hyperphagia and increased weight gain.

To further investigate the mechanisms underlying the obese phenotype induced by silencing ataxin-2 in the ARH, we evaluated a major hallmark of obesity, the insulin resistance. Others showed that alterations induced by obesity (or HFD) in the hypothalamus, liver and adipose tissue promote insulin resistance and this event aggravates the pathology in these organs (*Seoane-Collazo et al., 2015*).

We observe that mice under chow diet and with ataxin-2 silencing in the hypothalamus have insulin sensitivity decrease, mimicking the effects of a HFD. And as observed for body weight, ataxin-2 silencing in ARH in mice fed with HFD did not exacerbate insulin resistance already installed. Upon a short-term HFD feeding, compensatory mechanisms prevent additional damage to the metabolic status, however after a longer period of HFD consumption the compensatory mechanisms fail and occurs an exacerbation of the metabolic dysfunction (*Velloso and Schwartz, 2011*). After 4 weeks of HFD feeding, these compensatory mechanisms might prevent ataxin-2 silencing of further promoting metabolic dysfunction, hence the unchanged insulin sensitivity.

In the present study we also observed that ataxin-2 silencing in ARH of mice fed with chow diet increases pro-inflammatory cytokines, such as TNF-α, IL-1β and TGFβ1. This increase of pro-inflammatory cytokines could be the mechanism underlying the insulin resistance observed in mice.

Some studies show that the increase in pro-inflammatory cytokines in the hypothalamus is directly related to insulin resistance and the dysregulation in the central control of liver and adipose tissue (white and brown), which further potentiate insulin resistance (*Velloso and Schwartz, 2011; Valdearcos et al., 2015; Glass and Olefsky, 2016*). Moreover, over-nutrition and obesity increase pro-inflammatory factors such as TNF-α, IL-1β, IL-6 and SOCS3 levels in ARH (*Bjørbaek et al., 1998; Milansky et al., 2009; Thaler et al., 2012*). The increase of pro-inflammatory factors can disrupt NPY/AgRP and POMC neurons firing and impair food intake

regulation controlled by the neuropeptides system (*Thaler et al., 2012; Dalvi et al., 2016*). One relevant issue regarding pro-inflammatory cytokines increase is the fact that the increase of one promotes the increase of others, for example, IL-1β increases TNF-α and TGF1β in the hypothalamus (*Plata-Salaman and Ilyin, 1997*). TGF1β may act as anti-inflammatory or as pro-inflammatory (*Tsai et al., 2016*). Moreover, others also showed that TGFβ1 is increased in the hypothalamus on obesity and potentiates glucose metabolism disturbances (*Yan et al., 2014*). TGF1β activates NF-κB by induction of stress granules formation and sequestering elements required for NF-κB inhibition (*Yan et al., 2014*). The concerted increase in TGF1β observed upon ataxin-2 ARH silencing, might promote further damage to hypothalamic physiology.

Moreover, we observed that ataxin-2 silencing also increased SOCS3 mRNA expression, which was already described in obese models and related to leptin and insulin resistance (*Bjørbaek et al., 1998; Wunderlich et al., 2014*). Ataxin-2 hypothalamic silencing also increased GFAP mRNA levels, one marker of gliosis. This GFAP increase in the hypothalamus was also described by others in mice under HFD (*Horvath et al., 2010; Deake et al., 2011; Thaler et al., 2012; Buckman et al., 2013*) and it was also correlated to leptin and insulin resistance (*Hsuchau et al., 2009; Horvath et al., 2010; Schur et al., 2015*).

The increase of pro-inflammatory pathways can also impair the autonomic control of the hypothalamus over metabolic actions, contributing for liver steatosis, decreased BAT thermogenic ability and WAT inflammation with adipocyte hypertrophy and death (*Zheng and Lazar, 2013; Valdearcos, 2015; Seoane-Collazo et al., 2015; Lage et al., 2016*). Therefore, the hypothalamic inflammation that occurs in mice with ataxin-2 silencing might be the cause for the observed liver histological alterations and also for the insulin resistance. Further studies are needed to confirm this hypothesis. Insulin resistance and hepatic lipid accumulation function as a cycle, in one hand insulin resistance and hyperinsulinemia lead to hepatic lipogenesis and promote liver steatosis, while liver lipid accumulation promotes inflammation and endoplasmic reticulum stress further exacerbating insulin resistance (*Glass and Olefsky, 2016*). Even though we did not observe hyperinsulinaemia upon ataxin-2 silencing, it is already described that normal insulin signaling in a context of metabolic dysfunction, can also contribute to hepatic steatosis through deranged mTOR signalling (*Sun and Lazar, 2013*).

Ataxin-2 silencing promoted liver pathology in mice fed with chow diet and exacerbated liver pathology in HFD-fed mice. The observed modifications in the liver might be caused by the weight gain and insulin resistance, or by the hypothalamic dysfunction induced by ataxin-2 silencing. Nevertheless, given the fact that both groups with silenced hypothalamic ataxin-2

present alterations in liver, these results suggest that hypothalamic ataxin-2 is a player of liver metabolic homeostasis.

Beyond the liver, adipose tissue remodeling is one of the most direct effects of obesity. Ataxin-2 silencing in mice fed with chow diet increased WAT adipocytes diameter, in a similar way as HFD did. Ataxin-2 silencing in chow diet changed BAT structure and brown adipocytes acquired a phenotype more similar to white adipocytes, or in other words, BAT "whitening". BAT "whitening" occurs due to BAT hypoxia and the loss of mitochondria (that confer BAT a brown colour) leading to the decline in BAT thermogenic ability (*Shimizu and Walsh, 2015*). Decreased BAT function can potentiate weight gain and disrupt glucose metabolism (*Shimizu et al., 2014*). WAT increase and the putative BAT "whitening" caused by ataxin-2 hypothalamic can account for the overall metabolic alterations observed in this study, namely weight gain and insulin resistance. Furthermore, these observations suggest a role for ataxin-2 in the hypothalamic autonomic regulation of peripheral organs.

In conclusion, this study shows, for the first time, that lower levels of ataxin-2 in the hypothalamus increase body weight and mimic several aspects of obesity induced by HFD. This study corroborates the hypothesis that hypothalamic ataxin-2 influences energy balance, most likely acting through insulin signaling. Our observations suggest ataxin-2 as a potential target for the treatment or prevention of obesity and its metabolic consequences.

CHAPTER 5

Hypothalamic ataxin-2 overexpression prevents diet-induced obesity and insulin resistance

5.1. Abstract

The strong genetic component of obesity etiology is a challenge for the effectiveness of weight loss programs but is also an opportunity to the development of new targets for therapeutic approaches. ATXN2 gene, encoding a polyglutamine protein named ataxin-2, is localized in 12q24.1 within a well-documented obesity trait, the *ATXN2/SH2B3*. Moreover, ataxin-2 protein is involved in several cellular metabolic processes like lipid metabolism, insulin receptor endocytosis and nutrient sensing pathways. In the previous chapter we showed that mice under HFD have lower levels of ataxin-2 in the hypothalamus. Therefore, the aim of the present study was to investigate whether the reestablishment of hypothalamic ataxin-2 levels could prevent obesity diet-induced or ameliorate some metabolic changes associated to obesity.

The specific reestablishment of ataxin-2 in the ARH was achieved by a gene transfer approach delivering lentiviral vectors encoding for ataxin-2 directly in the ARH of mice by stereotaxic injection. After surgery, mice were fed either a chow diet or challenged with high fat diet (HFD) for 4 weeks. Throughout the study, body weight, food intake and insulin sensitivity were evaluated, as well as locomotor activity by behavioural tests. In the end of the study we studied the hypothalamus of these mice as well as peripheral metabolic organs (liver, white and brown adipose tissue).

The results show that ataxin-2 overexpression in the ARH prevented diet-induced obesity, without changes in food intake and motor activity. ARH ataxin-2 reestablishment prevented insulin resistance, hepatic lipid accumulation and decreased adipocyte size in white adipose tissue in mice. Furthermore, ataxin-2 overexpression was able to prevent hypothalamic inflammation induced by diet, decreasing pro-inflammatory markers in this brain region.

The ability of ataxin-2 to prevent diet-induced obesity phenotype suggest that ataxin-2 could be a potential new target for anti-obesity therapeutic strategies, but also related pathologies such as diabetes or non-alcoholic fatty liver disease. Moreover, this knowledge contributes to a better understanding of ataxin-2 role in metabolism regulation and the underlying mechanisms of metabolism and obesity development.

5.2 Introduction

Obesity affects more than 600 million people worldwide, numbers that only tend to increase in the next decades. Obesity, more specifically high waist circumference, the abdominal fat accumulation and ectopic fat accumulation in the liver, pancreas and heart, constitute major risk factors for the development of metabolic syndrome (*Alberti et al., 2006; Feldman et al., 2015*). Metabolic syndrome is characterized by the presence of hyperglycaemia, insulin resistance, dyslipidaemia and high blood pressure and represents a major risk factor for the development of chronic debilitating disorders such as type 2 diabetes and cardiovascular diseases (*Timar et al., 2000; Ritchie et al., 2007; Han and Lean, 2016*).

Genome wide association studies (GWAS) have associated several single nucleotide polymorphisms (SNP) with increased body mass index (BMI), fat distribution and risk for obesity and metabolic syndrome (*Lu and Loos, 2013; Albuquerque et al., 2015*). The first SNP associated with increased BMI was mapped to the *FTO* (fat mass and obesity associated) gene in 2007 (*Frayling et al., 2007*). Genetic variants as *FTO* SNP may interfere with weight loss programs, providing a higher impact in body weight than diet, exercise and even surgery (*Xiang et al., 2016*). GWAS not only provided new insight on the genetic components of obesity, but also identified potential new players in energy balance regulation. FTO functions as a nutritional sensor (*Cheung et al., 2013*). Furthermore, FTO is highly expressed in the arcuate nucleus of the hypothalamus and the modulation of FTO in this region can impact food intake and body weight (*Tung et al., 2010*).

GWAS associated the *ATXN2/SH2B3* trait in chromosome 12, with obesity and metabolic disorders (*Li et al., 2004; Chagnon et al., 2004; Dong et al., 2005; Auburger et al., 2014*). In obese children, authors observed a common pattern in the CAG repeat structure and a frequent SNP in ATXN2 gene (*Figueroa et al., 2009*). ATXN2 mutation is associated with a neurodegenerative disorder, Spinocerebellar ataxia type 2 (SCA2) (*Pulst et al., 1996; Sanpei et al., 1996; Sahba et al., 1998*). And, interestingly, all individuals in a family of SCA2 patients were found to be polyphagic and obese (*Abdel-Aleem and Zaki, 2008*). Moreover, SNPs in ataxin-2 binding protein 1 (A2BP1) gene, a functional mediator of ataxin-2 that contributes to normal function of ATXN2, were also correlated with obesity (*Ma et al., 2010; Dong et al., 2015*).

In addition, ataxin-2 knockout mice (ATXN2 KO) are heavier and have some metabolic dysfunctions, such as dyslipidaemia and insulin resistance (*Kiehl et al., 2006; Lastres-Becker et al., 2008b; Meierhofer et al., 2016*).

Several obesity-related genes, like FTO, act as nutritional sensors through hypothalamic signalling (*Tung et al., 2010; Cheung et al., 2013; Speakman, 2015*). The hypothalamus is the brain region responsible for body weight and energy balance maintenance, lipid metabolism and insulin signaling (*Bisschop et al., 2014; Waterson and Horvath, 2015; Sohn et al., 2015*). We sought out to understand if ataxin-2 could potentially be itself a good candidate to an obesity-related gene.

In the previous chapter, we showed that mice under HFD have lower levels of ataxin-2 in the hypothalamus. Therefore, the aim of the present study was to investigate whether the reestablishment of hypothalamic ataxin-2 levels could prevent the increase of food intake and body weight caused by HFD, and prevent the metabolic dysfunction phenotype related to obesity such as insulin resistance.

5.3. Results

ARH ataxin-2 overexpression prevents diet-induced obesity without changing food intake

We overexpressed ataxin-2 in mice arcuate nucleus of the hypothalamus (ARH) using lentiviral vectors. As controls, we injected mice with lentiviral vectors encoding for GFP. Then we divided these two groups of mice in two subgroups, one fed with chow diet and the other fed with HFD for 4 weeks.

Hypothalamic ataxin-2 overexpression had no effect on body weight in mice fed with chow diet, however, upon high fat diet (HFD) it significantly prevented the diet-induced obesity (Figure 5.1A, B). Interestingly, the reduced body weight gain was not accompanied by a decrease in food intake as it could be somehow expected (Figure 5.1C), which could indicate a role of hypothalamic ataxin-2 in the regulation of energy expenditure.

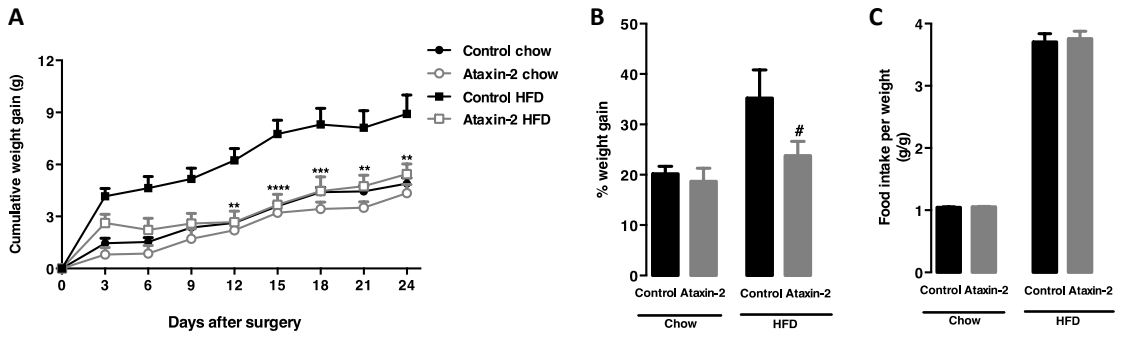

Figure 5.1. Ataxin-2 overexpression in the ARH prevents diet-induced obesity (A, B) without changes food intake (C). Control mice were injected with lentivirus encoding for GFP and the treated groups were injected with a lentivirus encoding for ataxin-2. (A) Cumulative weight gain (in g) and (B) percentage of weight gain of mice fed with chow and high fat diet (HFD) for 4 weeks with ARH ataxin-2 overexpression. (C) Total food intake (4 weeks) expressed in a ratio with animal weight (g/g) for ataxin-2 silencing group. Control chow n=7-9; Ataxin-2 chow n=5; Control HFD n=3-5; Ataxin-2 HFD n=10. Data is expressed as the mean ± SEM. **p<0.01, ***p<0.001, ****p<0.0001 compared to control HFD for the respective time point; $^{\#}p$<0.05 relative to control HFD.

Hypothalamic ataxin-2 overexpression does not change hypothalamic neuropeptide expression neither motor activity

Body weight maintenance is a tightly regulated process that relies on the balance between food intake and energy expenditure. Food intake increase with low energy expenditure can

induce obesity, while increased motor activity and/or energy expenditure can promote weight loss. Comparing mice under HFD with or without hypothalamic ataxin-2 overexpression mice, there was no difference on food intake (Figure 5.1C) neither increase in motor activity (Figure 5.2B, C) that could explain the lower body weight of mice with hypothalamic ataxin-2 overexpression. In all 4 groups, no differences were found in the overall activity, neither in the total distance travelled nor in the mean velocity (Figure 5.2B, C).

Since ataxin-2 hypothalamic overexpression prevented diet-induced weight gain without reducing food intake or changing activity, we next analyzed the expression of hypothalamic neuropeptides responsible for the food intake regulation. Consistently with the observation of no alterations in food intake, ataxin-2 overexpression in the hypothalamus had no effect on mRNA levels of orexigenic (AgRP and NPY) or anorexigenic (POMC) neuropeptides (Figure 5.2A).

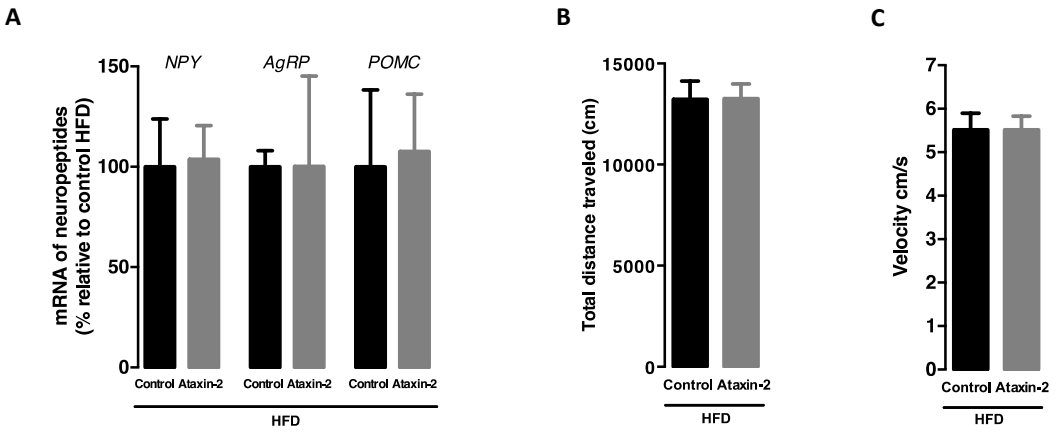

Figure 5.2. Mice under HFD with ataxin-2 overexpression in ARH did not show differences in mRNA levels of orexigenic (NPY and AgRP) and anorexigenic (POMC) hypothalamic neuropeptides (A) neither in motor activity (B) compared to control mice (under HFD). Mice were fed with HFD for 4 weeks. As controls, mice were injected with GFP in ARH. (A) The mRNA levels in mice hypothalamus were measured by RT-qPCR, as described in Chapter 3. Control HFD n=5; Ataxin-2 HFD n=5-7. Data is expressed as the mean ± SEM. (B, C) Open field behavioural test in HFD group, control versus ataxin-2 overexpression. The physical activity was measured by Open field test, as described in Chapter 3. (B) Total distance travelled (cm) and (C) mean velocity (cm/s). Control HFD n=5; Ataxin-2 HFD n=10. Data is expressed as the mean ± SEM.

Ataxin-2 overexpression in the ARH promotes improves insulin sensitivity in HFD fed mice

Insulin resistance is part of many pathophysiological dysfunctions in the metabolic syndrome. It can result directly from hypothalamic dysfunction, but also from the overall compromise of peripheral metabolic organs such as liver and adipose tissue (*Velloso and Schwartz, 2011; Glass and Olefsky, 2016*). Based on previous evidences reporting ataxin-2 as an insulin sensitivity mediator (reviewed in *Carmo-Silva et al., 2017*), and the observation that ataxin-2 overexpression in the hypothalamus prevent diet-induced obesity, we next investigated whether overexpression of ataxin-2 in ARH changes insulin resistance development induced by HFD, by measuring glycaemia and performing insulin tolerance test (ITT).

Interestingly, even though ataxin-2 overexpression in the ARH prevented weight gain in HFD, it had no effect in glycaemia in both groups (Figure 5.3A). However, ataxin-2 hypothalamic reestablishment significantly improved glucose clearance in the HFD group (showing also a trend in the chow group) (Figure 5.3B, C). These results suggest that hypothalamic ataxin-2 is an insulin action modulator.

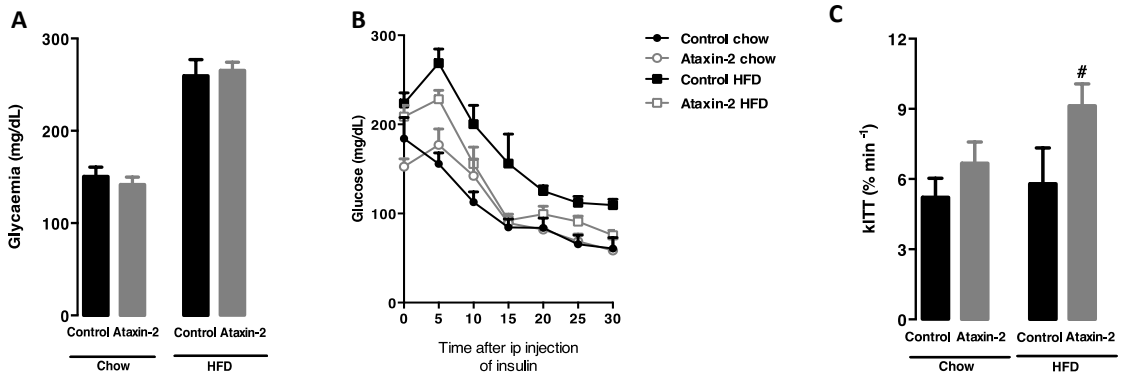

Figure 5.3. Ataxin-2 overexpression in the ARH promotes improves insulin sensitivity (B, C) in HFD fed mice, with no changes in glycaemia (A). (A) Glycaemia (mg/mL) in mice fed with chow and high fat diet in the ataxin-2 silencing groups. (B) Insulin tolerance test (ITT) in chow and high fat diet in silencing group, expressed as the glycaemia (mg/mL) measured every 5 min after intraperitoneal injection of insulin. (C) kITT representing the glucose clearance rate (% *per* min) in chow and high fat diet for both control and ataxin-2 ARH overexpressing group. Control chow n=7-9; Ataxin-2 chow n=5; Control HFD n=3; Ataxin-2 HFD n=6. Data is expressed as the mean ± SEM. $^{\#}p<0.05$ relative to control HFD.

Hypothalamic ataxin-2 overexpression prevents liver pathology caused by HFD

Hepatic lipid accumulation induced by HFD can compromise physiological liver functions like glucose metabolism and insulin sensitivity (*Birkenfeld and Shulman, 2014*). We investigated the effect of ataxin-2 overexpression on lipid accumulation in liver, using Hematoxilin and Eosin (H&E) staining (Figure 5.4A)

Ataxin-2 overexpression (Figure 5.4A) in chow diet group had no significant impact on liver histology. As expected, control mice under HFD, that developed obesity, showed liver lipid accumulation (Figure 5.4A). Hypothalamic ataxin-2 overexpression in mice under HFD prevented liver pathology induced by HFD (Figure 5.4A). In fact, the liver histology in ataxin-2 overexpression mice fed with HFD was very similar to chow diet control mice. Furthermore, ataxin-2 overexpression also improved the lipid profile, as triglycerides and total cholesterol levels were decreased, in mice fed with HFD (Figure 5.4B, C).

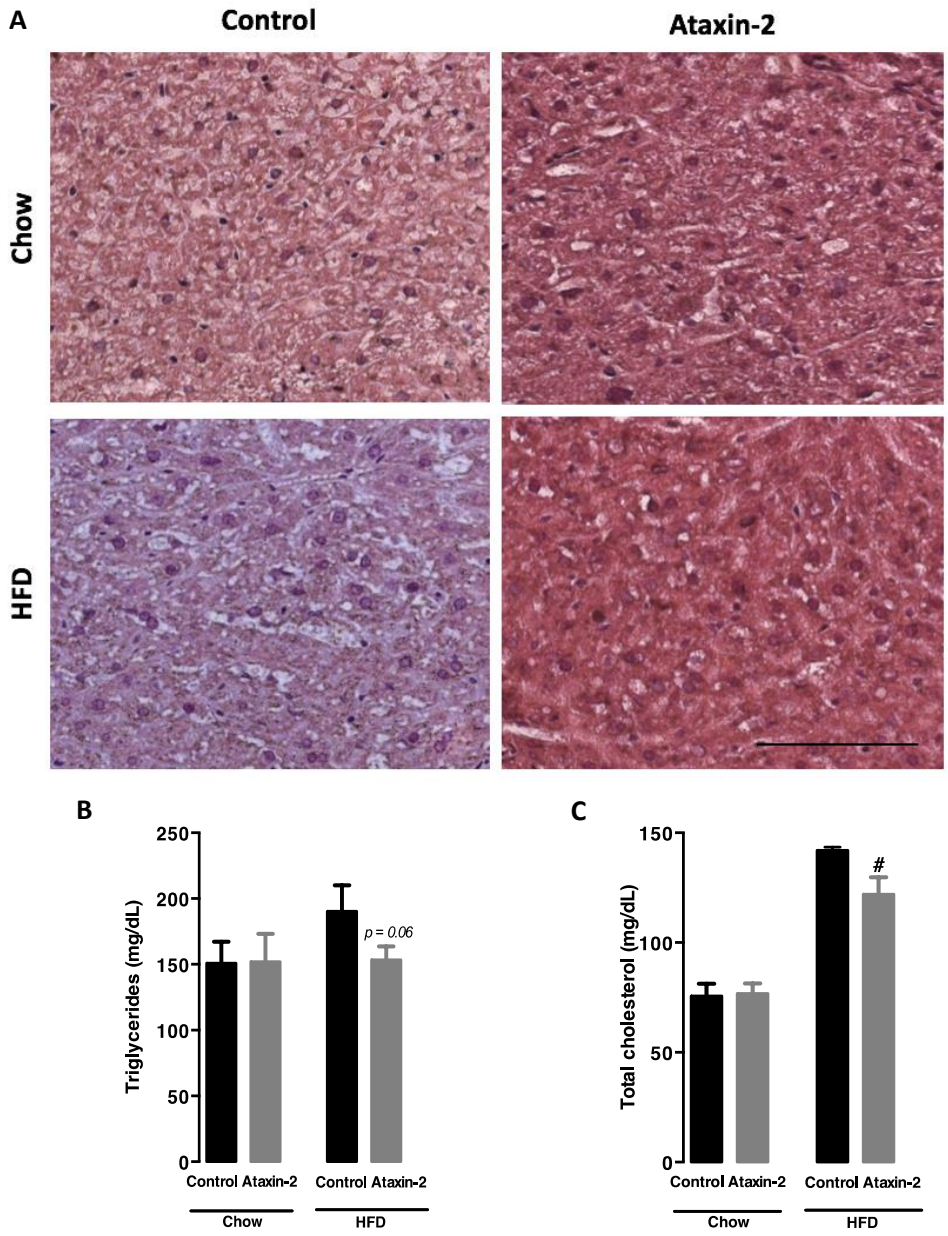

Figure 5.4. Ataxin-2 overexpression in ARH prevents hepatic lipid accumulation (A) and decreases triglycerides (B) and cholesterol levels (C). (A) H&E stained sections from livers of mice fed either chow or HFD for 4 weeks, in the ataxin-2 overexpressing groups. For each group n=3-4, scale bar 100 µm. (B) Triglycerides (mg/mL) content in serum of mice fed with chow and high fat diet in the ataxin-2 overexpressing groups. (C) Total cholesterol (mg/mL) in the serum of mice fed with chow and high fat diet in the ataxin-2 overexpressing groups. Control chow n=7-9; Ataxin-2 chow n=5; Control HFD n=3; Ataxin-2 HFD n=6. Data is expressed as the mean ± SEM. $^{\#}p<0.05$ relative to control HFD.

ARH ataxin-2 overexpression prevents adipose tissue alterations induced by HFD

Upon a fat enriched-diet and obesity, white adipose tissue (WAT) adipocytes enlarge and infiltration of immune cells in WAT can occur. While WAT is mainly responsible for energy,

brown adipose tissue (BAT) is responsible for energy expenditure through the regulation of thermogenesis (*Smitka and Marešová, 2015; Gómez-Hernández et al., 2016*). Some evidence also show that BAT modulates hypothalamic regulation of energy balance (*Contreras et al., 2014; Lage et al., 2016*). WAT plasticity, related to the ability of white adipocytes to turn into bright (or beige) adipocytes, presents as new putative therapeutic strategy of obesity (*Giordano et al., 2016*).

To investigate the WAT and BAT changes in the four group of mice, we stained WAT and BAT samples with H&E (Figure 5.5A). Under HFD, as expected, WAT have increased adipocyte size (Figure 5.5A, B). Moreover, hypothalamic ataxin-2 reestablishment was able to promote a decrease in the mean area of adipocytes (Figure 5.5B).

Obesity decreases vascularity and induces hypoxia in BAT, which can lead to the process of "whitening" of the brown adipocytes (*Shimizu and Walsh, 2015*). And others already showed that HFD increases BAT adipocytes with "white" phenotype characterized by enlarged adipocytes with enlarged lipid droplets, a process called "BAT whitening" (*Shimizu et al., 2014*). As expected, we also observed that BAT from mice under HFD presented enlarged adipocytes with bigger lipid droplets (Figure 5.5C). Interestingly, BAT from mice under HFD with ataxin-2 hypothalamic overexpression have fewer large brown adipocytes and the BAT phenotype was more similar to control chow diet (Figure 5.5C).

Overall these results suggest that hypothalamic ataxin-2 overexpression in HFD does not only prevent diet-induced weight gain but also the consequent peripheral dysregulation of the adipose tissue.

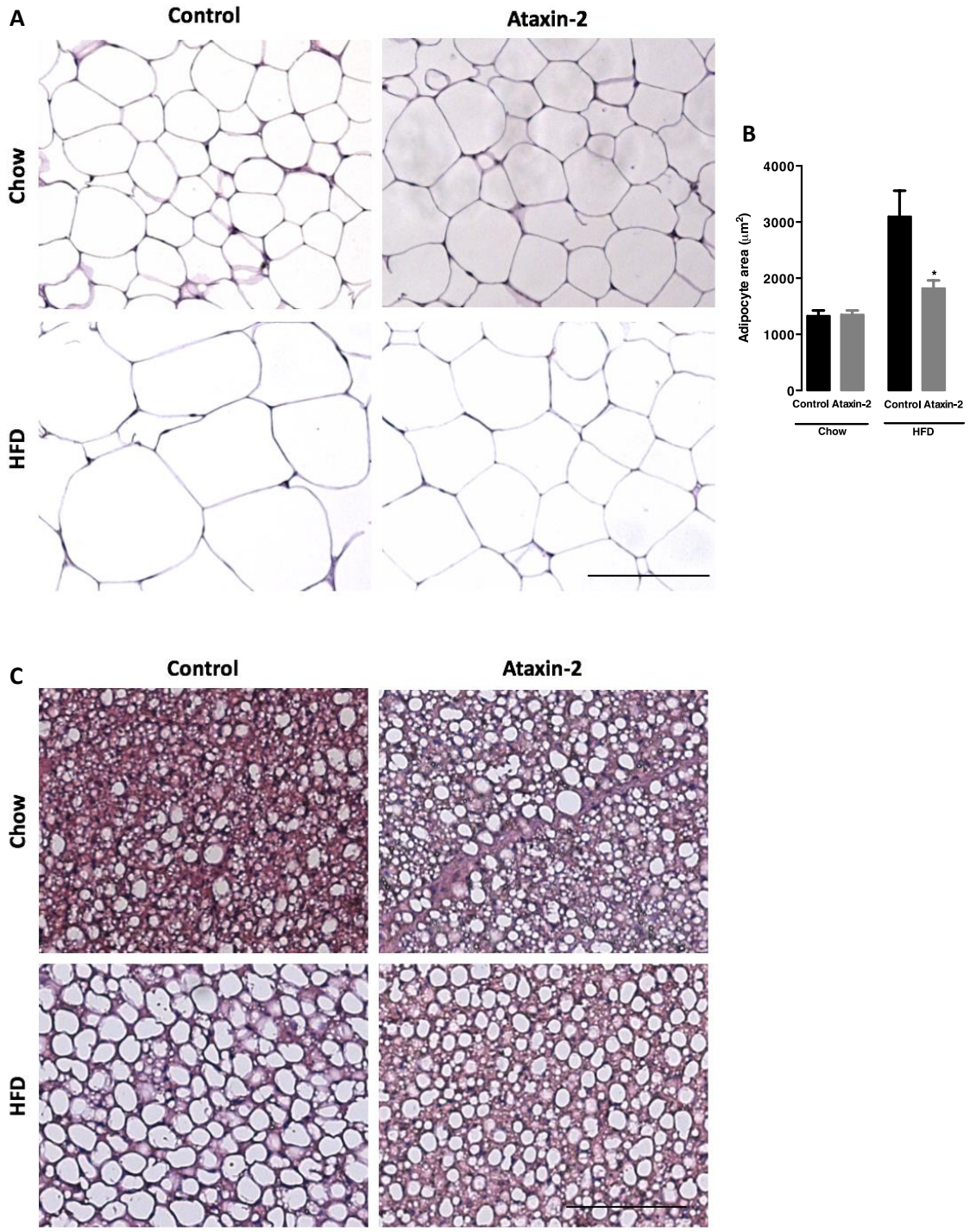

Figure 5.5. Hypothalamic ataxin-2 overexpression prevented WAT (A, B) and BAT (C) alterations induced by HFD. (A) H&E stained sections from white adipose tissue (WAT) of mice fed either chow diet (chow) or HFD for 4 weeks. For each group n=3-4, scale bar 100 μm. (B) Adipocyte diameter upon chow and HFD in control and ataxin-2 overexpressing groups. For each group n=3-4. (C) H&E stained sections from interscapular brown adipose tissue (BAT) of mice fed either with chow or HFD for 4 weeks. For each group n=3-4, scale bar 100 μm. Data is expressed as the mean ± SEM. $^{*}p<0.05$ relative to control HFD.

ARH ataxin-2 overexpression prevents diet-induced hypothalamic inflammation

HFD promotes hypothalamic inflammation; with some authors stating that one-day of HFD consumption is enough to promote the inflammatory insult (*Thaler et al., 2012; Waise et al., 2015*). Pro-inflammatory stimuli can lead to hypothalamic neuronal networks dysfunction promoting an overall metabolic dysregulation. Interestingly, hypothalamic inflammation is not only observed in obesity but also in other metabolic disorders such as anorexia or cancer-related cachexia (*Zhang et al., 2008; Pimentel et al., 2014; Tsai et al., 2016; Dalvi et al., 2016; Burfeind et al., 2016*). Thus, we investigated if hypothalamic ataxin-2 overexpression changed the characteristic hypothalamic inflammation induced by HFD.

As observed by others, HFD increased the expression of mRNA of pro-inflammatory markers TNF-α (238 $\pm$ 56.7 % of chow, Figure 5.6A), IL-1β (405.6 $\pm$ 183.3 % of chow, Figure 5.6B), TGFβ1 (162.3 $\pm$ 47.4 % of chow, Figure 5.6C) and marker of astrogliosis, GFAP (192.3 $\pm$ 43.4 % of chow, Figure 5.6D) in the hypothalamus.

Hypothalamic ataxin-2 overexpression prevented HFD induced inflammation as observed by the decrease in pro-inflammatory cytokines mRNA expression TNF-α (29.8 $\pm$ 9.4 % of control, Figure 5.6E), IL-1β (13.2 $\pm$ 3.3 % of control, Figure 5.6F), TGFβ1 (50.1$\pm$ 3.9 % of control, Figure 5.6G). Moreover, it also prevented astrogliosis, observed by a decrease in GFAP mRNA expression (42 $\pm$ 10.7 % of control, Figure 5.6H). Altogether these data show that ataxin-2 overexpression prevents hypothalamic inflammation induced by HFD.

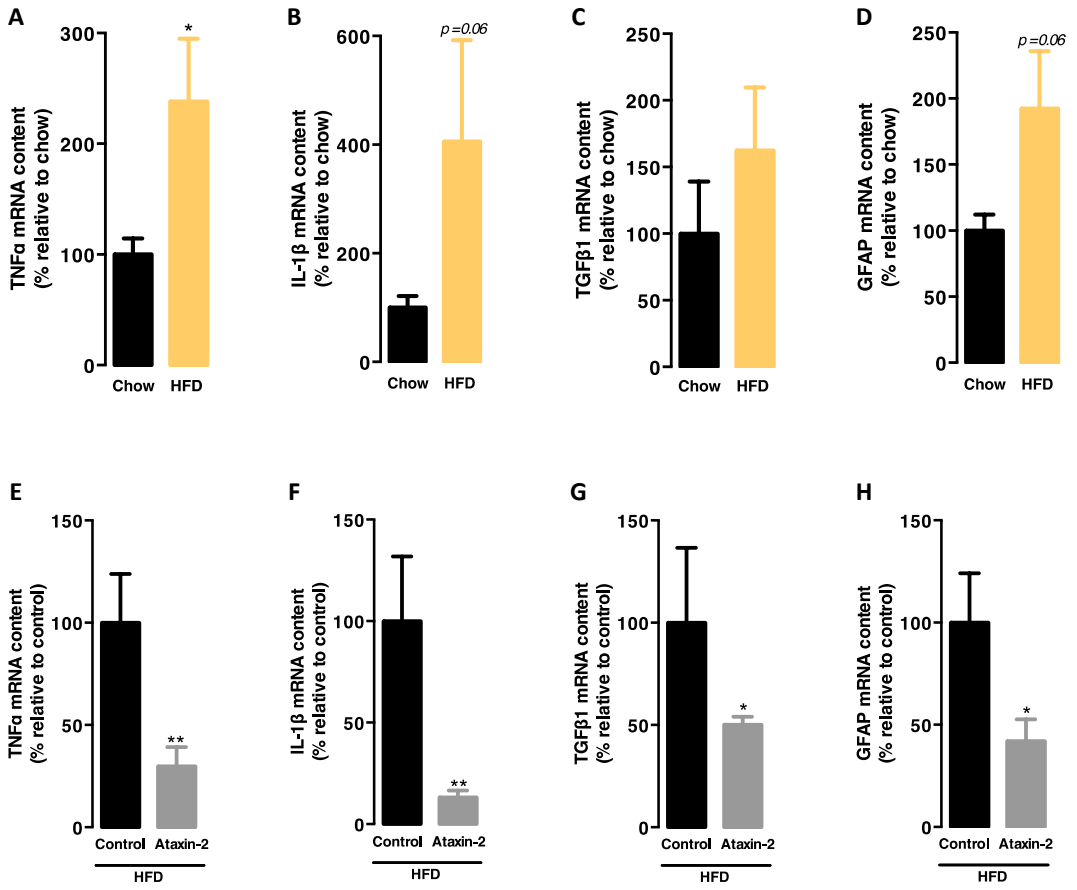

Figure 5.6. High fat diet increases pro-inflammatory markers (A-C) and astrogliosis (D) in the hypothalamus. Ataxin-2 ARH specific reestablishment decreases inflammatory (E-G) and astrogliosis (H) markers induced by HFD. (A-D) Increased hypothalamic mRNA levels of inflammatory and astrogliosis markers in mice fed with HFD versus control mice fed with chow. (A) Tumor necrosis factor alpha, (B) Interleukin 1 Beta, (C) Transforming growth factor Beta 1, (D) Glial fibrilary acidic protein mRNA content relative to control. (E-H) Decreased hypothalamic mRNA levels of inflammatory and astrogliosis markers in mice fed with HFD with ARH ataxin-2 overexpression. (E) Tumor necrosis factor alpha, (F) Interleukin 1 Beta, (G) Transforming growth factor Beta 1, (H) Glial fibrilary acidic protein mRNA content relative to control. Control chow n=3; Control HFD n=3-5; Ataxin-2 HFD n=5-7. Data is expressed as the mean ± SEM. *p<0.05 and **p<0.01, compared to respective control (A-D: control chow; E-H: control HFD).

5.4. Discussion

In the present study we showed that ataxin-2 overexpression in the hypothalamus can prevent diet-induced obesity and insulin resistance. Ataxin-2 overexpression in HFD mice resulted in a similar phenotype to chow-fed mice.

We observed that ataxin-2 hypothalamic overexpression had no effect on food intake in chow diet or in HFD group. Therefore, the weight gain prevention due to ataxin-2 overexpression was not promoted by a reduction in the caloric intake. Interestingly we also did not detect any alterations in physical activity, that could result in increased energy expenditure. Moreover, ataxin-2 ARH overexpression did not change hypothalamic orexigenic (AgRP and NPY) nor anorexigenic (POMC) peptides mRNA levels, which might support the unaffected feeding behaviour observed. However, the effect of HFD on hypothalamic orexigenic and anorexigenic neuropeptides levels and how they are modulated upon treatment is still controversial (*Bergen et al., 1999; Lemus et al., 2015*). Our data suggests that neither food intake reduction nor increased motor activity are mechanisms responsible by which ataxin-2 hypothalamic overexpression prevents body weight gain of mice under HFD. Further studies are necessary to scrutinize the exact mechanism through which hypothalamic ataxin-2 alters body weight gain.

Insulin sensing by the hypothalamus drives the regulation of peripheral metabolic organs (*Seoane-Collazo et al., 2015; Thon et al., 2016*). It is known that HFD promotes hypothalamic damage that further aggravate the overall metabolic condition, namely insulin resistance (*Valdearcos et al., 2015*). In this study we observed that hypothalamic ataxin-2 overexpression significantly prevented HFD-induced insulin resistance, with no changes on glycaemia. Others already showed that insulin resistance might occur as consequence of several mechanisms, and without, or before, hyperglycaemia. For example, insulin resistance can be a consequence of several cellular damage mechanisms like lipotoxicity, oxidative stress, ER stress or the alteration in metabolic signaling pathways (*Boucher et al., 2014*). The improvement in insulin sensitivity upon hypothalamic ataxin-2 reestablishment might derive from the weight prevention and its effect on peripheral organs like the liver and the adipose tissue.

It is known that hypothalamus modulates the sympathetic regulation of liver, BAT and WAT (*Cornejo et al., 2016; Devarokonda and Mobbs, 2016; Lage et al., 2016*). The ARH is responsible for the regulation of hepatic processing and storage of energy in the form of triglycerides, for instance, orexigenic neuropeptides can promote the release of triglycerides (*Bruinstroop et al., 2014*). Taking this information into account, we investigated the putative beneficial effect of hypothalamic ataxin-2 overexpression on liver and adipose tissues pathology induced by HFD.

In the present study, we observed a tendency for a decrease in serum triglycerides levels upon hypothalamic ataxin-2 overexpression, compared to the control group also fed with HFD. This improvement in hepatic functioning was also observed in the liver histology analysis. HFD increased accumulation of lipid droplets in the liver tissue, which was almost completely prevented by the ataxin-2 ARH overexpression. Furthermore, ataxin-2 hypothalamic overexpression also prevented hypercholesterolemia caused by HFD. These results are in accordance with previous studies that suggest that ataxin-2 is involved in lipid processing (*Lastres-Becker et al., 2008b; Fittschen et al., 2015; Meierhofer et al., 2016*). Thus, the hypothalamic ataxin-2 overexpression might prevent diet-induced dyslipidaemia. These interesting results could be also related to the weight gain prevention, but can also suggest a role for ataxin-2 in hypothalamic on liver control.

Adipose tissue inflammation, WAT enlargement and BAT "whitening" are some of the effects of obesity and HFD. Moreover, recent studies show that WAT and BAT are relevant contributors (not only effectors) of hypothalamic regulation of energy balance (*Gomez-Hernandez et al., 2016; Lage et al., 2016*). In the present study we observed that ataxin-2 hypothalamic overexpression was able to prevent adipocyte enlargement upon HFD and in some extent the "whitening" of BAT. BAT is responsible for energy expenditure promoting thermogenesis, others showed that HFD induces changes namely BAT "whitening" (*Shimizu et al., 2014*). On the opposite, knockdown the orexigenic neuropeptide NPY promotes thermogenesis and induce WAT "browning" (*Criori et al., 2011; Chao et al., 2011; Seoane-Collazo et al., 2015; Valdearcos et al., 2015).* As HFD impairs BAT responsiveness, hypothalamic ataxin-2 overexpression might prevent BAT deregulation hence promoting thermogenesis and energy expenditure, which results in lower body weight gain. This may suggest a possible role of ataxin-2 on BAT β adrenergic receptors activation. However, this hypothesis needs further studies.

Hypothalamic inflammation is also a hallmark of obesity and HFD consumption (*Cai, 2013; Cavadas et al., 2016*). Hypothalamic inflammation can compromise *per se*, energy balance and the hypothalamic regulation of peripheral organs (*Valdearcos et al., 2015*). Others showed that after only one day of HFD the levels of some pro-inflammatory cytokines increase in the hypothalamus (*Thaler et al., 2012; Waise et al., 2015*). This increase in inflammatory markers can still be observed after one and four weeks under HFD (*Thaler et al., 2012; de Araújo et al., 2016*). In our study, after four weeks of HFD, we observed an increase in inflammation markers (TNF-α, IL-1β, TGFβ1) and in astrogliosis marker (GFAP) in the hypothalamus, which was prevented by hypothalamic ataxin-2 overexpression. The amelioration of hypothalamic

inflammation observed upon ataxin-2 overexpression might be the cause for the improvements observed in hepatic lipid content and BAT physiology.

Altogether the results of the present study support the role of ataxin-2 as a metabolic player. Moreover, ataxin-2 modulation in the hypothalamus directly changes body weight and energy homeostasis, making the gene ATXN2 a putative new candidate as a gene related to obesity. In addition, further studies are needed to explore whether ataxin-2 modulation could be a new therapeutic strategy for obesity and its related metabolic consequences.

CHAPTER 6

Hypothalamic ataxin-2 is a mediator of insulin sensitivity

6.1. Abstract

Insulin resistance is the major driver for type 2 diabetes and determinant for the pathophysiology of several metabolic disorders. The hypothalamus, as a major metabolic sensor, is promptly affected by damages in insulin signalling. Hypothalamic insulin resistance compromises the autonomic control over organs such as the liver and adipose tissue, further promoting metabolic damage. Finding new players in hypothalamic physiology affecting insulin sensitivity is a determinant step in finding new therapeutic targets for type 2 diabetes. Ataxin-2 KO (ATXN2 KO) mice are obese and insulin resistant, presenting also other metabolic alterations like dyslipidaemia and hepatosteastosis. Moreover, ataxin-2 protein participates in the endocytosis of tyrosine kinase receptors such as InsR, and mediates metabolic pathways involved in insulin signalling like the mTOR. These observations suggest that ataxin-2 might play an important role in insulin sensitivity. However, the central effect of ataxin-2 over insulin sensitivity was never studied.

The aim of this study was to investigate the impact of the re-establishment of ataxin-2 in the hypothalamus of ATXN2 KO mice in insulin sensitivity and overall metabolic status. For this, we overexpressed ataxin-2 in the arcuate nucleus of the hypothalamus of ATXN2 KO mice, and maintained the animals in high fat diet for eight weeks.

Ataxin-2 restoration promoted an increase in insulin sensitivity and the improvement of peripheral hepatic pathology, without changes in body weight. Ataxin-2 hypothalamic overexpression had no effect in food intake of ATXN2 KO mice. In the present study, we observed that ATXN2 KO mice are hyperactive and have a lower food intake when compared to wild type littermates. Despite this phenotype, ATXN2 KO mice are obese. We demonstrate for the first time that disruptions in the circadian rhythm of ATXN2 KO mice might be responsible for the obesity and insulin resistance. We observed that ataxin-2 hypothalamic overexpression in ATXN2 KO mice rescues the expression of altered clock genes.

The global knockout of ataxin-2 promotes obesity and insulin resistance. Here we provide evidences that ataxin-2 impacts insulin sensitivity in a hypothalamic-mediated manner. The present results not only propose a new role for ataxin-2 in the hypothalamus but also provide evidences that targeting hypothalamic ataxin-2 might be a new potential therapeutic approach for insulin resistance conditions.

6.2. Introduction

Insulin resistance is an integrative part of the metabolic syndrome and the driver of type 2 diabetes. Obesity is tightly related with hyperinsulineamia and insulin resistance (*Kahn and Flier, 2000; Kahn et al., 2006*). Moreover, insulin resistance has proved to be the link between obesity and other disorders such as cardiovascular diseases, infertility and cancer (*Brunning et al., 2000; Mokdad et al., 2003; Ruiz-Nunez et al., 2016; Tahergorabi et al., 2016*).

Insulin signalling in the CNS controls peripheral fat and glucose metabolism. Insulin regulates hepatic glucose production (HPG), adipose tissue lipolysis and whole-body insulin sensitivity (*Obici et al., 2002; Koch et al., 2008; Scherer et al., 2011; Heni et al., 2014; Gancheva and Roden, 2016*). In the brain, namely in the hypothalamus, insulin signalling promotes negative energy balance by suppressing food intake and promoting energy expenditure (*Benoit et al., 2002; Clegg et al., 2003; Brown et al., 2006*). The hypothalamic alterations caused by high fat diet and obesity, such as inflammation, ER stress, mitochondrial dysfunction and overall loss of proteostasis (*Cavadas et al., 2016*) are promoters of insulin resistance. In one hand, these mechanisms compromise metabolic pathways that control insulin sensitivity, in other, the dysregulation of insulin signalling in the brain will compromise the hypothalamic autonomic regulation of liver and adipose tissue, further exacerbating insulin resistance (*Zancu et al., 2011; Konner and Brunning, 2012; Gancheva and Roden, 2016*).

ATXN2 KO mice are obese, insulin resistant and present marked dyslipidaemia (*Lastres-Becker et al., 2008b*), making this mouse model an excellent platform to study the effect of ataxin-2 in a metabolic impairment setting. ATXN2 KO mice present increased insulin production and hyperinsulinemia, which are features of an obesity scenario. Moreover, these mice present alterations in the insulin receptor (InsR) processing, with an increase of InsR mRNA in the brain and liver, and a decrease in the protein levels (*Lastres-Becker et al., 2008b*). Ataxin-2 participates in the endocytic internalization of tyrosine kinases receptors such as InsR (*Nonis et al., 2009; Drost et al., 2013*), thus the absence of ataxin-2 in the KO mice might be responsible for the defective insulin signalling.

The mTOR signalling pathway is tightly connected to insulin resistance (*Yoon and Choi, 2016*). In this context, branched chained amino acids (BCAA), that are increased in obesity and type 2 diabetes, are thought to be responsible for the overactivation of mTOR, which impairs insulin signalling (*Newgard et al., 2009: McCormack et al., 2013; Lackey et al., 2013*). A recent study observed disturbances in BCAA metabolism in ATXN2 KO (*Meierhofer et al., 2016*). Indeed, ATXN2 ortholog in yeast Pbp1, was found sequestrate yeast TORC1 to stress granules upon low

cellular energy, inhibiting mTOR pathway (*Takahara and Maeda, 2012; Buchan et al., 2012; DeMille et al., 2015*). More recently, ataxin-2 was found to modulate leucine/mTOR- driven phosphorylation of ribosomal protein S6 (RPS6) (*Fittschen et al., 2015*) and to be modulated upon mTOR inhibition by rapamycin (*Lastres-Becker et al., 2016*). Furthermore, the absence of ataxin-2 in MEFs of ATXN2 KO mice promotes the increased phosphorylation of RPS6K, a substrate for mTOR signalling (*Lastres-Becker et al., 2016*). According to this scenario, in ATXN2 KO mice, the absence of ataxin-2 could result in the defective sequestration of TORC1 and the overactivation of the mTOR signalling cascade. This might account not only for the increased growth (obesity), but also for the insulin resistance observed in these mice.

Given the metabolic dysfunction of ATXN2 KO mice and the recent evidences that point to a modulatory role of ataxin-2 in metabolic pathways, we aimed to understand how hypothalamic ataxin-2 could influence the overall metabolic status. For this purpose, we overexpressed ataxin-2 in the hypothalamus of ATXN2 KO mice and evaluated parameters altered in this genotype, namely body weight, food intake, insulin sensitivity, dyslipidaemia and motor behaviour. We also performed behavioural tests, namely the open field, and assessed the mRNA of clock genes. Furthermore, we used an *in vitro* model of insulin resistance in hypothalamic neurons (palmitate stimulation in the cell line of mouse embryonic hypothalamic neurons mHypo-N42) to evaluate role of ataxin-2 in mTOR signalling.

6.3. Results

Re-establishment of ataxin-2 in the ARH in global ATXN2 KO mice has no effect on body weight

As already mentioned, the ATXN2 KO mice show higher weight gain when fed with HFD when compared to WT littermates (*Lastres-Becker et al., 2008b*). In the present study we hypothesized that this phenotype was due to the absence of ataxin-2 in the hypothalamus. To test this hypothesis, we re-established ataxin-2 levels in the hypothalamus of ATXN2 KO mice using lentiviral vectors. Additionally, heterozygous mice were used as a control of ataxin-2 dosage expression. The different mice groups were fed with a HFD in order to recapitulate previous studies (*Kiehl et al., 2006; Lastres-Becker et al., 2008b*). Concordant with previous studies (*Kiehl et al., 2006; Lastres-Becker et al., 2008b; Scoles et al., 2012*), the ATXN2 KO mice presented a higher weight gain compared to wild-type littermates when fed a 45 % HFD (Figure 6.1A, B). Also the heterozygous mice showed higher % of weight gain when compared to WT littermates (Figure 6.1B). However, the hypothalamic ataxin-2 overexpression in the ATXN2 KO mice did not promote body weight changes compared to the control KO mice (GFP injected) (Figure 6.1A, B).

The studies that evaluated food intake of ATXN2 are controversial (*Kiehl et al., 2006; Lastres-Becker et al., 2008b; Scoles et al., 2012*). In our study using the rodent diet with 45% kcal% fat D12451 from Research diets, we observed that ATXN2 KO mice and of heterozygous mice eat less then wild-type mice (Figure 6.1C). Moreover, ataxin-2 hypothalamic overexpression in ATXN2 KO did not change food intake, when compared to control ATXN2 KO mice.

ATXN2 KO mice and heterozygous mice eat less but have higher weight gain compared to WT littermates, which might suggest other type of metabolic dysregulations. These results show that body weight gain observed in ATXN2 KO mice is not caused by an increase in food intake.

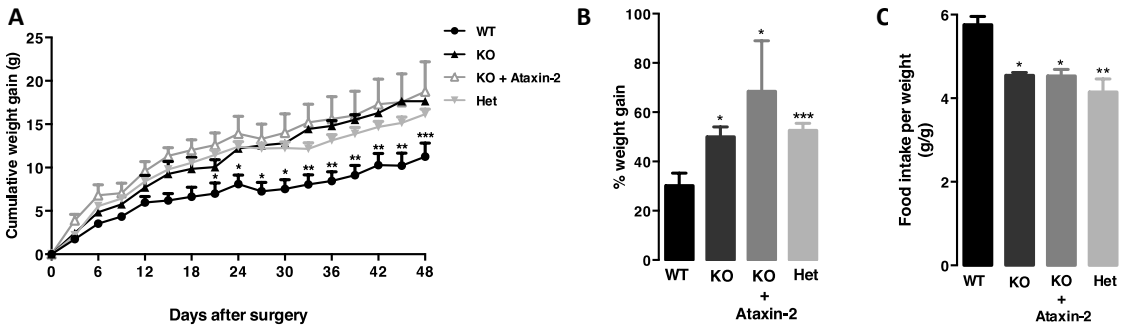

Figure 6.1. Hypothalamic ataxin-2 overexpression in ATXN2 KO mice does not change body weight gain (A) neither food intake (C). Wild type (WT), ATXN2 KO mice (KO, KO + ataxin-2) and heterozygous mice (Het) were fed with HFD for 8 weeks. Mice were injected, in the ARH, with a Lentivirus encoding for GFP (KO group) or lentivirus encoding to Ataxin-2 (KO + ataxin-2) were injected in ARH. Heterozigous mice (Het) were used as a representative control of moderated ataxin-2 expression. (A) Cumulative weight gain (in g) throughout 8 weeks. **p*<0.05, ***p*<0.01, ****p*<0.001 compared to ATXN2 KO mice. (B) Percentage of body weight gain of mice fed with high fat diet for 8 weeks. (C) Total food intake (8 weeks) expressed by animal weight (g/g). WT n=5; KO n=2; KO+Atx2 n=3; Het n=5. Data is expressed as the mean ± SEM. **p*<0.05, ***p*<0.01 and ****p*<0.001 compared to control WT.

Re-establishment of ataxin-2 in the ARH in global ATXN2 KO mice improves insulin sensitivity

Other studies showed that ATXN2 KO are insulin resistant although without hyperglycaemia (*Lastres-Becker et al., 2008b*). Moreover, other studies show that hypothalamus regulates insulin sensitivity (*Clegg et al., 2003*). In order to investigate if hypothalamic ataxin-2 overexpression changes insulin sensitivity of ATXN2 KO mice, we evaluated glycaemia levels and performed insulin tolerance test (ITT).

We observed that ATXN2 KO mice had higher glycaemia levels when compared to WT littermates (Figure 6.2A). Interestingly, ATXN2 KO mice with hypothalamic overexpression of ataxin-2 have decreased glycaemia levels compared to the KO control mice, with levels similar to the heterozygous mice (Figure 6.2A).

Regarding insulin response, ATXN2 KO mice presented an impaired glucose clearance relative to wild-type control (Figure 6.2B, C). Ataxin-2 ARH overexpression ameliorated the insulin resistance as observed by an increase in the glucose clearance rate (kITT) compared to the KO control mice (Figure 6.2C).

Overall these results suggest that hypothalamic ataxin-2 is relevant to hypothalamic regulation of insulin action. Thus, ataxin-2 absence in the hypothalamus might contribute to insulin resistance observed in ATXN2 KO mice.

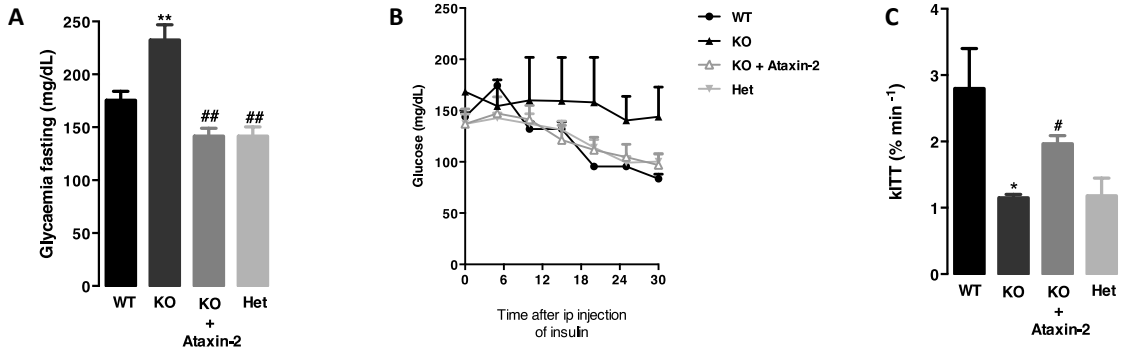

Figure 6.2. Hypothalamic ataxin-2 overexpression in ATXN2 KO mice rescues hyperglycaemia and improves insulin sensitivity. Wild type (WT), ATXN2 KO mice (KO, KO + ataxin-2) and heterozygous mice (Het) were fed with HFD for 8 weeks. Mice were injected, in the ARH, with a Lentivirus encoding for GFP (KO group) or lentivirus encoding to Ataxin-2 (KO + ataxin-2) were injected in ARH. Heterozigous mice (Het) were used as a representative control of moderated ataxin-2 expression. (A) Glycaemia (mg/mL) in all groups of mice fed with high fat diet. (B) Insulin tolerance test (ITT) in wild type mice (WT), ATXN2 KO with (KO + ataxin-2) or without (KO) ARH ataxin-2 overexpression and heterozygous mice, expressed as the glycaemia (mg/mL) measured every 5 minutes after intraperitoneal injection of insulin. (F) kITT representing the glucose clearance rate (% *per* min) for all groups tested. WT n=5; KO n=2; KO + ataxin-2 n=3; Hets n=5. Data is expressed as the mean ± SEM. *p<0.05 and **p<0.01 compared to control WT, $^{\#}p$<0.05 and $^{\#\#}p$<0.01 compared to KO.

Hypothalamic ataxin-2 overexpression in ATXN2 KO mice improves peripheral metabolic organs

Others showed that ATXN2 KO mice present hepatic lipid accumulation (*Kiehl et al., 2006; Lastres-Becker et al., 2008b*), which might be a result of obesity and insulin resistance. As expected, we also observed hepatic lipid accumulation in ATXN2 KO mice, when compared to wild-type littermate (Figure 6.3A). Interestingly, ataxin-2 overexpression in the ARH of ATXN2 KO mice rescued liver lipid accumulation (Figure 6.3A), similarly to what is observed in the heterozygous mice (Figure 6.3A).

Regarding WAT, since the KO mice gain more weight than WT, as expected, we observed a trend for increase in adipocytes (Figure 6.3B). Ataxin-2 overexpression in the ARH of ATXN2 KO mice promoted a trend for reduced adipocyte size (Figure 6.3B), however the reduced number of animals used in this experiment does not allow for quantification and conclusive results.

The ATXN2 KO mice present brown adipose tissue (BAT) with adipocytes with bigger lipid droplets ("whiter" BAT) when compared to BAT from WT (Figure 6.3C), a feature of obesity and metabolic dysfunction. Ataxin-2 overexpression in the hypothalamus of the KO mice was not sufficient to completely rescue BAT structure (Figure 6.3C).

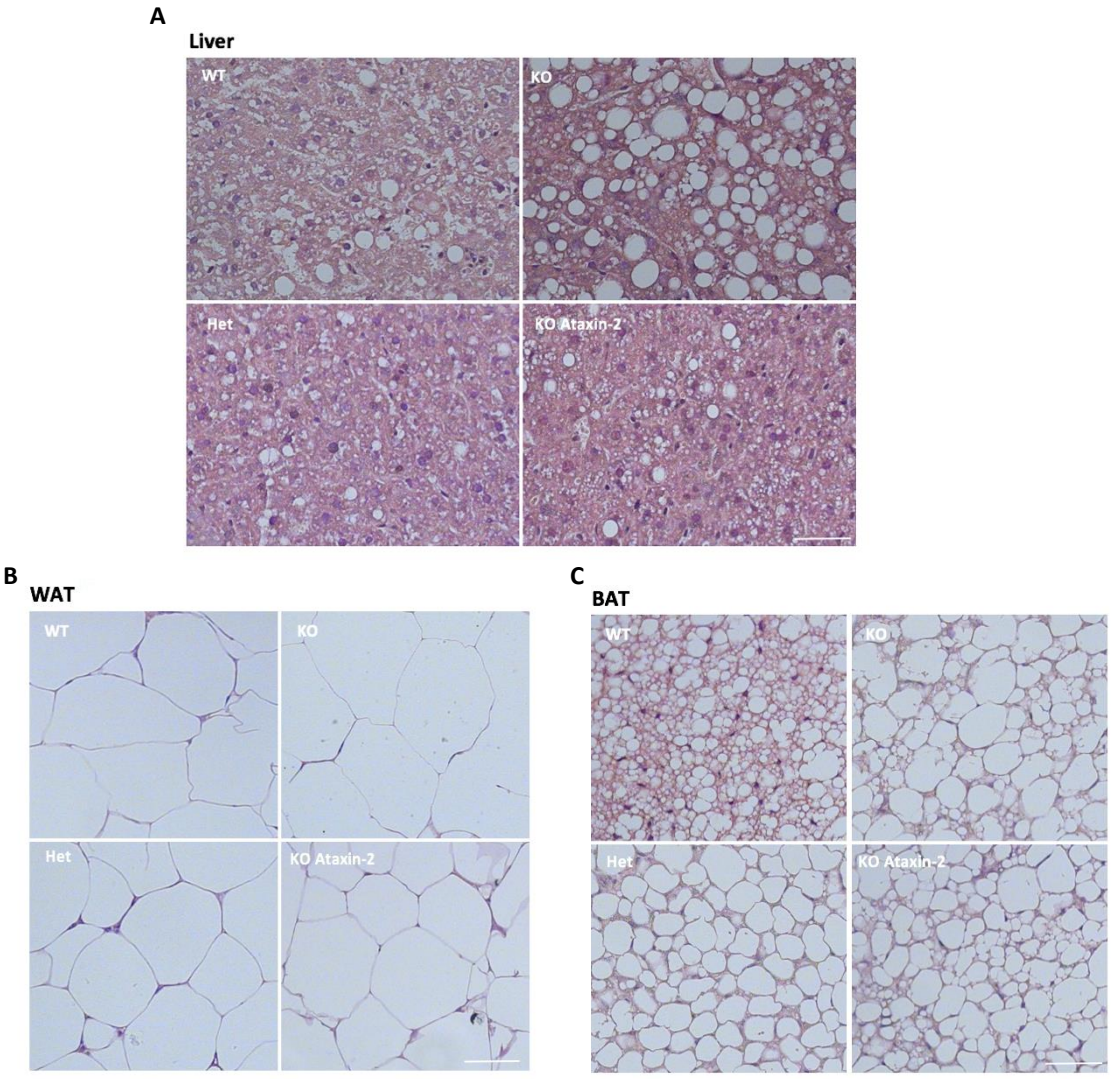

Figure 6.3. Hypothalamic ataxin-2 overexpression in KO mice promotes improvements in peripheral metabolic organs. Mice were injected, in the ARH, with a Lentivirus encoding for GFP (KO group) or lentivirus encoding to Ataxin-2 (KO + ataxin-2) were injected in ARH. Heterozigous mice (Het) were used as a representative control of moderated ataxin-2 expression. (A) H&E staining of liver sections of wild type (WT), heterozygous (Het), ATXN2 KO mice and ATXN2 KO mice with hypothalamic ataxin-2 overexpression (KO + ataxin-2) fed with HFD for 8 weeks. (B) H&E staining of white adipose tissue (WAT) sections from all groups of mice fed with HFD for 8 weeks. (C) H&E staining of brown adipose tissue (BAT) sections of all groups of mice fed with HFD for 8 weeks. WT n=5; KO n=2; KO+Atx2 n=3; Het n=3. Scale bar 50 μm.

ATXN2 KO mice show different motor activity pattern

Others showed previously that ATXN2 KO mice have increased spontaneous locomotor activity in the open field test, suggesting that a decrease in physical activity does not contribute to the obese phenotype observed in these mice (*Lastres-Becker et al., 2008b*).

In order to evaluate if the locomotor activity was changed in the animals in the same conditions, we performed open field test in all mice groups at the end of the study. We performed the behaviour assessment twice, one time during the inactive period (light), and other during the active period (dark). Interestingly, we observed an increase in locomotor activity in ATXN2 KO mice when compared to the WT littermates, in the inactive period testing (light, or day period) (Figure 6.4A). Both groups of ATXN2 KO (ATXN2 KO and ATXN2 KO + ataxin-2) mice show an increase in mean distance and velocity compared to the WT littermates during the inactive period (light, or day period) (Figure 6.4B, C).

ATXN2 KO mice with hypothalamic ataxin-2 overexpression appear to have reduced tracing of locomotor activity in both periods when compared to the ATXN2 KO control mice (Figure 6.4A). However, these differences are not translated in statistic differences in the mean distance and velocity (Figure 6.4B, C) between the two groups in both periods. However, ataxin-2 hypothalamic overexpression reduced distance and velocity in the active period (dark) in ATXN2 KO mice when compared to WT littermates (6.4B, C).

A normal pattern of mice behaviour is represented by a higher motor activity in the dark period corresponding to their active phase, comparative to the light period (Figure 6.4A). In fact, we observed that WT mice are more active during the dark period compared to light period (Figure 5.4A). This increased activity is translated in a significant increase in distance travelled (272 $\pm$ 15.3 % of inactive period, p<0.0001) and velocity (274 $\pm$ 15.4 % of inactive period, p<0.0001) in the active period (dark) relative to inactive period (light, 100%) (Figure 6.4D, E). However, in the KO mice the difference observed between inactive and active phase is much smaller both in distance (ATXN2 KO: 173.2 $\pm$ 11.3 % of inactive period, p<0.05; ATXN2 KO + ataxin-2: 127 $\pm$ 3.9 % of inactive period, p<0.05) and velocity (ATXN2 KO: 170 $\pm$ 11.3 % of inactive period, p<0.05; ATXN2 KO + ataxin-2: 127 $\pm$ 4.3 % of inactive period, p<0.05) (Figure 6.4D, E). These results suggest that KO mice might be hyperactive as they do not follow the normal circadian behaviour, meaning that these mice are more active during the light phase and do not change their behaviour in the dark cycle. In the heterozygous mice, no differences were observed between behaviour during active and inactive phase, also indicating a deregulation in behaviour.

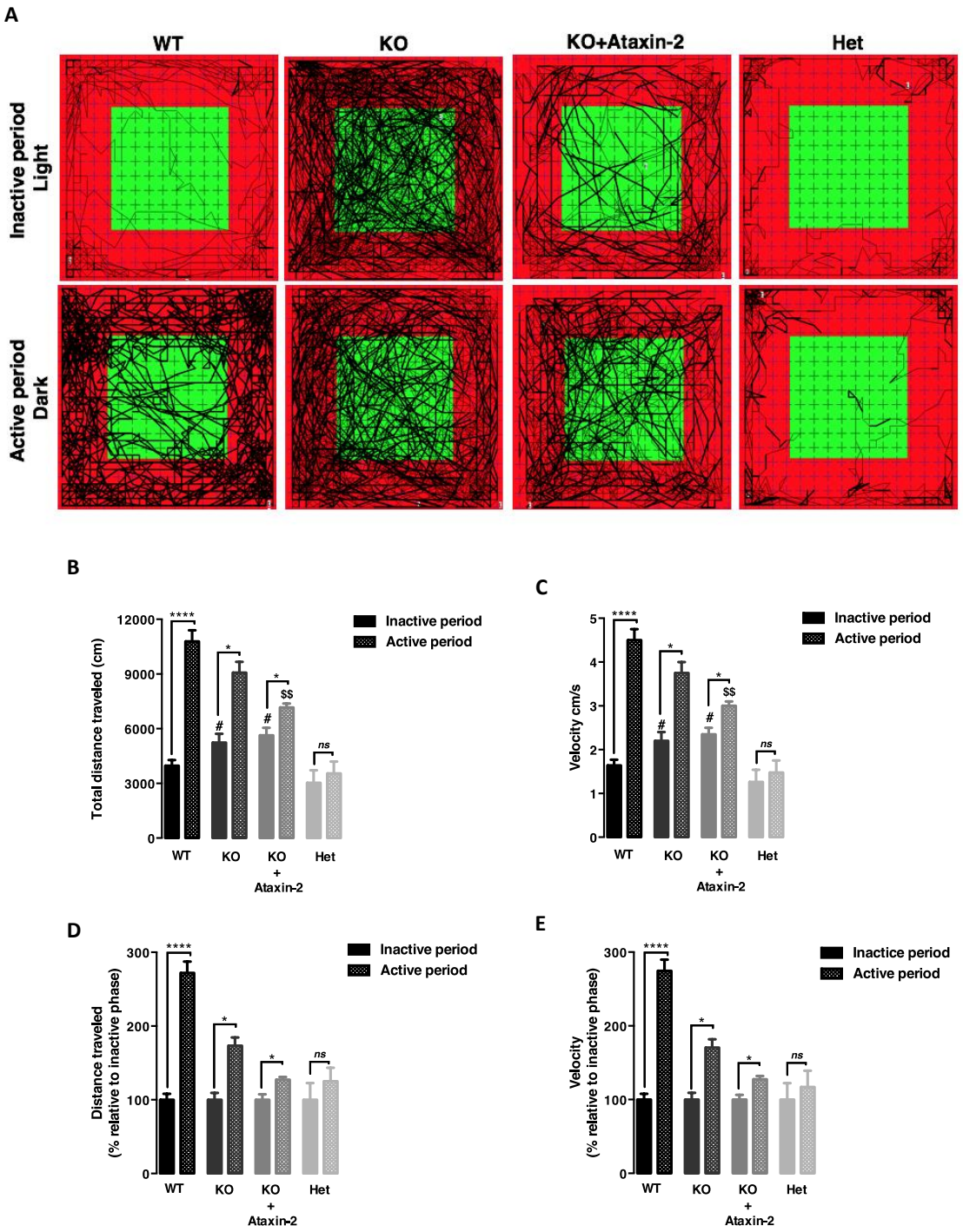

Figure 6.4. ATXN2 KO mice display alterations in the motor activity in the active and inactive period. Mice were injected, in the ARH, with a Lentivirus encoding for GFP (KO group) or lentivirus encoding to Ataxin-2 (KO + ataxin-2) were injected in ARH. Heterozigous mice (Het) were used as a representative control of moderated ataxin-2 expression. Open field test to evaluate motor activity in all groups of mice (WT, KO, KO + Ataxin-2, Het) fed with HFD for 8 weeks in the inactive (light) and active (dark) periods. Bars with full colour represent the inactive period whereas patterned bars represent the active period. (A) Activity tracing of the two zones analyzed in the Open field test for all groups in both active and inactive periods. Tracing obtained by the analysis of the ActiTrack software. (B) Total distance traveled (cm) in

the Open field test in all groups for light and dark period. (C) Mean velocity (cm/s) in the 40 min duration of the Open field test. (D) Fold change of total distance traveled in the Open field test in all groups, relative to the inactive period. (C) Fold change of mean velocity in the Open field test in all groups, relative to the inactive period. WT n=5; KO n=2; KO + ataxin-2 n=2; Heterozygous n=5. Data is expressed as the mean ± SEM. *p<0.05 and ****p<0.0001 relative to respective group on "Inactive period". *p<0.05 relative to "WT in inactive period". $^{\$\$}p$<0.01 relative to "WT in active period".

ATXN2 KO mice have different clock genes expression pattern

The intrinsic circadian rhythms are controlled by a transcription-translation feedback system that involves core genes like the Circadian Locomotor Output Cycles Kaput (CLOCK), Aryl hydrocarbon receptor nuclear translocator-like protein 1 (ARNLT or Bmal1), Cryptochrome (Cry), Period 1 (Per1) and Period 2 (Per2) (*Jin et al., 1999; Reppert and Weaver, 2002; Gooley et al., 2003*). Disruption in all of these genes or just in one may change circadian behaviour that (*Eckel-Mahan and Sassone-Corsi, 2013; Challet, 2015; Coomans et al., 2015*). Since we observed alteration in circadian activity of ATXN2 KO mice we evaluated the expression of the circadian clock genes (Figure 6.5).

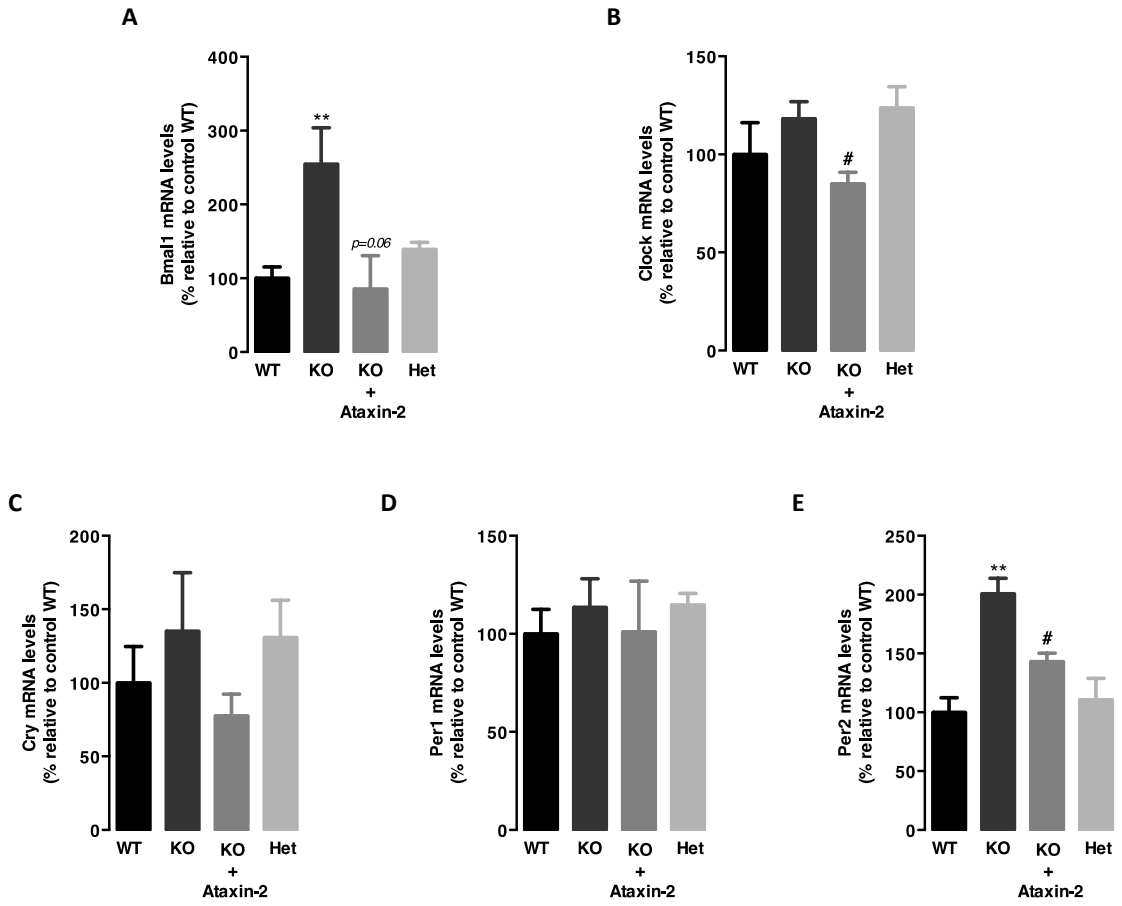

Figure 6.5. ATXN2 KO mice have higher levels of clock genes (Bmal1 and Per2) mRNA and the hypothalamic ataxin-2 overexpression reverts these changes. Mice were injected, in the ARH, with a Lentivirus encoding for GFP (KO group) or lentivirus encoding to Ataxin-2 (KO + ataxin-2) were injected in ARH. Heterozigous mice (Het) were used as a representative control of moderated ataxin-2 expression. The mRNA content of clock genes Bmal1 (A), Clock (B), Cry (C) Per1 (D), Per2(E) in whole hypothalamus of Wild Type (WT), ATXN2 KO mice (KO), ATXN2 KO mice with overexpression of ataxin-2 in the ARH (KO + Ataxin-2) and ataxin-2 heterozygous mice (Het). WT n=5; KO n=2; KO+Atx2 n=3; Het n=3. Data is expressed as mean ± SEM. **p*<0.05 and ***p*<0.01 compared to control WT, $^{\#}p$<0.05 compared to KO.

No statistically significant differences were observed in mRNA levels of Cry and Per1 in the four groups of animals (Figures 6.5C, D). However, we observed that hypothalamus of ATXN2 KO mice have significant higher levels of mRNA of *Bmal1* (254.5 ± 49.4 % of control, Figure 6.5A) and *Per2* (207.7 ± 13.1 % of control, Figure 6.5E), compared to WT mice. Interestingly, ataxin-2 ARH overexpression in the ATXN2 KO mice was able to significantly decrease mRNAs levels of *Bmal1* (85.5 ± 45 % of control, Figure 5.5A), *CLOCK* (84.9 ± 6 % of control, Figure 6.5B) and Per2 (143.1± 26 % of control, Figure 6.5E), to similar levels observed in WT control.

Others showed that a disruption in clock genes is directly correlated with insulin resistance and obesity (*Shi et al., 2013; Coomans et al., 2015; Ribas-Latre and Eckel-Mahan, 2016*). The re-establishment of Bmal1 and CLOCK expression observed in mice with overexpression ataxin-2 in ARH suggest that the reestablishment in the expression of these genes could be contributing to increased insulin sensitivity observed upon ataxin-2 overexpression in the KO mice.

ATXN2 KO mice have decreased levels of hypothalamic AgRP mRNA levels

Body weight results from the balance between food intake and energy expenditure. We observed that although ATXN2 KO mice eat less than WT mice (Figure 6.1C) and have higher physical activity (Figure 6.4), these animals are obese (Figure 6.1A, B). In order to further investigate the mechanisms underlying this paradox phenotype, we evaluated the hypothalamic neuropeptides involved in food intake, namely the expression of orexigenic neuropeptides such as Neuropeptide Y (NPY) and Agouti-related protein (AgRP) and the anorexigenic neuropeptide pro-opiomelanocortin (POMC). The unbalance between these neuropeptides expression is often associated with obesity (*Waterson and Horvath, 2015; Webber et al., 2015*). Interestingly, ATXN2 KO mice present a significant decrease in the orexigenic neuropeptide *AgRP* (58.4 $\pm$ 8.5% of control WT, Figure 6.6A) when compared to WT littermates. Interestingly, ataxin-2 overexpression in ARH of ATXN2 KO mice reverted AgRP decrease. Even though the mRNA levels of other neuropeptides were not statistically significant different between the groups of animals (Figure 6.6B, C), the tendency of changes suggest that in ATXN2 KO mice, hypothalamic expression of neuropeptides is concordant with the significant reduction in food intake.

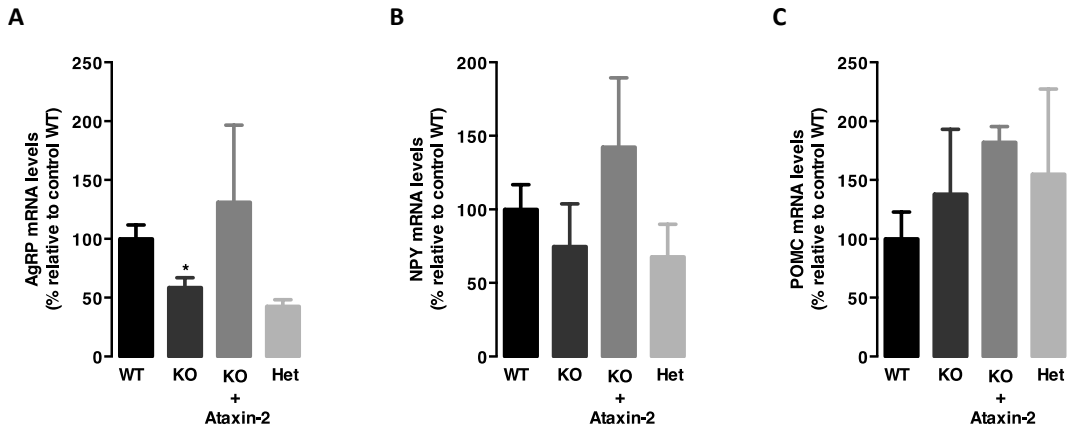

Figure 6.6. ATXN2 KO mice have lower levels of hypothalamic neuropeptide AgRP and overexpression of ataxin 2 in the hypothalamus reverts AgRP decrease. Mice were injected, in the ARH, with a Lentivirus encoding for GFP (KO group) or lentivirus encoding to Ataxin-2 (KO+ataxin-2) were injected in ARH. Heterozigous mice (Het) were used as a representative control of moderated ataxin-2 expression. Hypothalamic mRNA levels of orexigenic (AgRP and NPY) and anorexigenic (POMC) neuropeptides in the whole-hypothalamus in all groups of mice fed with HFD. (A) AgRP, (B) NPY and (C) POMC mRNA content relative to control WT. WT n=5; KO n=2; KO+Ataxin-2 n=3; Het n=3. Data is expressed as the mean ± SEM. **p*<0.05 compared to control WT.

Ataxin-2 hypothalamic re-establishment in KO mice mediates insulin signalling

As mentioned before ATXN2 KO mice have higher serum insulin and present insulin resistance (*Lastres-Becker et al., 2008b*). In the present study we observed that hypothalamic re-establishment of ataxin-2 in ATXN2 KO mice increased insulin sensitivity (Figure 6.3B, C). Although we did not observe an increase in serum insulin in the ATXN2 KO mice when compared to WT, the results show that ataxin-2 hypothalamic overexpression tendency decrease insulin levels in the ATXN2 KO mice (Figure 6.7A).

Lastres-Becker described that ATXN2 KO mice have lower levels of *InsR* mRNA expression in liver and cerebellum (*Lastres-Becker et al., 2008b*). In the present study, we evaluated *InsR* mRNA levels in the hypothalamus. We observed that ataxin-2 KO mice present lower levels hypothalamic *InsR* mRNA (15.1 ± 7.1% of control Figure 6.7B), when compared to WT littermates. And, ataxin-2 hypothalamic overexpression seem to rescue InsR downregulation observed in ATXN2 KO mice (53.7 ± 16.3% of control Figure 5.7B), although not statistically significant. KO mice also present hypothalamic depletion in the expression of Insulin receptor substrate 1 (IRS1) comparing to WT littermates (61.4 ± 13% of control Figure 6.7C). Once again, hypothalamic ataxin-2 seem also to revert *IRS1* decrease in KO mice (130.3 ± 53% of control Figure 6.7C). The observation that ATXN2 KO mice have lower levels of *InsR* and *IRS1* further supports the observation that insulin signalling is impaired in ATXN2 KO mice, which might be promoting the insulin resistance. The fact that hypothalamic ataxin-2 re-establishment can directly promote the increased expression of determinant factors of insulin signalling, suggests a significant role of ataxin-2 in insulin sensitivity and may explain the improved insulin response observed in the KO + ataxin-2 mice.

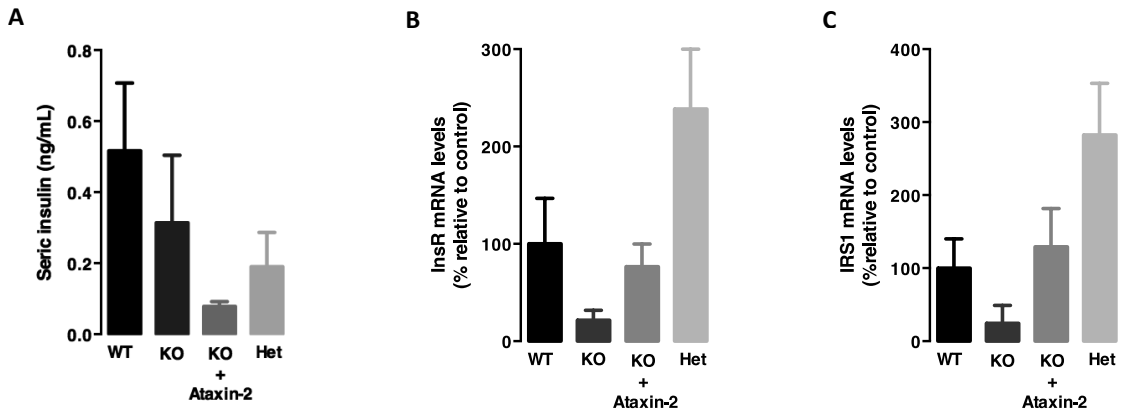

Figure 6.7. Ataxin-2 in the hypothalamus might impact insulin signaling pathway. Mice were injected, in the ARH, with a Lentivirus encoding for GFP (KO group) or lentivirus encoding to Ataxin-2 (KO + ataxin-2) were injected in ARH. Heterozigous mice (Het) were used as a representative control of moderated ataxin-2 expression. (A) Seric insulin levels (ng /mL) measured by ELISA in mice fed with HFD for 8 weeks. (B, C) mRNA content of factors involved in insulin signalling in the hypothalamus, Insulin receptor (InsR) (B) and Insulin receptor substrate 1 (IRS1) from the whole hypothalamus of mice in the KO experiment. WT n=5; KO n=2; KO + Ataxin-2 n=3; Het n=3. Data is expressed as the mean ± SEM.

Ataxin-2 modulation in vitro changes mTOR-signaling pathway

Since mTOR signalling pathway can directly affect insulin sensitivity (*Boucher et al., 2016; Yoon and Choi, 2016*) and some studies suggest that ataxin-2 regulate this metabolic pathway (*Fittschen et al., 2015; DeMille et al., 2015; Lastres-Becker et al., 2016*), we next evaluated the impact of ataxin-2 modulation of in mTOR activation. For that, we used a cell line of mouse hypothalamic neurons (mHypoN42) and treated them with palmitate, which is a saturated fatty acid used to promote compromises insulin signalling in hypothalamic neurons (*Riccardi et al., 2004; Mayer and Belsham, 2010*). Palmitate incubation decreased ataxin-2 protein levels (70.1 $\pm$ 14 % of control, Figure 6.8B) of mHypoN42.

In order to understand how ataxin-2 modulates mTOR pathway in an insulin resistance context, hypothalamic cells were transfected with ataxin-2 or with a shRNA targeting ataxin-2. As expected, palmitate increased mTOR (199.7 $\pm$ 30.7 % of control, Figure 6.8B) and RPS6K (145 $\pm$ 9.6% of control Figure 6.8C) activation. On the other hand, ataxin-2 overexpression was able to prevent this activation upon palmitate stimulation, lowering mTOR (118.5 $\pm$ 24.1 % of control, Figure 5.4.8B) and RPS6K (101.2 $\pm$ 2 % of control, Figure 5.4.8C) to basal control levels. Ataxin-2 silencing did not significantly change mTOR, probably because there is already an installed palmitate-induced ataxin-2 reduction.

Overall, these results suggest that hypothalamic ataxin-2 is a mediator the mTOR pathway and this might be the cause for some of the metabolic impairments observed in mice lacking ataxin-2, such as obesity and insulin resistance.

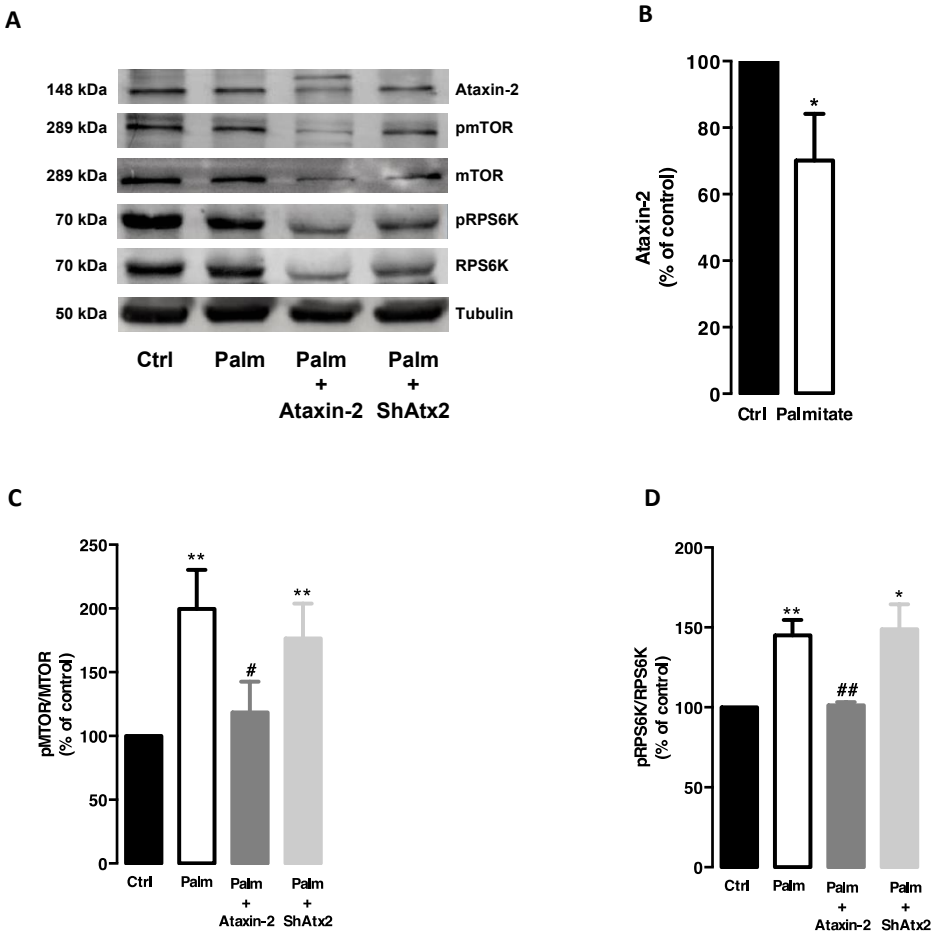

Figure 6.8. Ataxin-2 modulation *changes* mTOR signaling pathway in hypothalamic cells. mHypoN42 hypothalamic neurons were treated with vehicle/BSA as control (Ctrl) or 500 μM of Palmitate for 24h (Palm). Cells were transfected with Ataxin-2 (to overexpress ataxin-2) or ShAtaxin-2 (to silence ataxin-2) plasmids and then treated with 500 μM of palmitate (24 h). (A-D) Whole-cell extracts were assayed for ataxin-2, phospho-mTOR (p-mTOR), total mTOR (mTOR), phospho-RPS6K (p-RPS6K), RPS6K and tubulin immunoreactivity by Western blotting. n = 6 Data is expressed as the mean ± SEM. *p< 0.05 and **p< 0.01 significantly different from control; $^{\#}p$<0.05 and $^{\#\#}p$<0.001 significantly different from palmitate condition.

6.4. Discussion

Global ataxin-2 knockout promotes obesity and other metabolic alterations like insulin resistance, hepatosteatosis and dyslipidaemia (*Kiehl et al., 2006; Lastres-Becker et al., 2008b*). These events share some common mechanisms and are all part of the complex picture that is metabolic dysfunction. All these conditions influence and worsen each other; therefore, it is difficult to discern causes from consequences. The increase in the knowledge regarding mechanisms involved in these dysfunctions and new mediators of these processes can open the way to anti-obesity therapies. In this work, we tried to unravel the contribution of ataxin-2 in metabolic regulation by assessing its role in hypothalamic physiology.

As expected, ATXN2 KO mice in HFD gained more weight when compared to their wild type littermates. However, this increased weight gain was not caused by an increase in food intake. In fact, ATXN2 KO mice present a significant decrease in food intake relative to WT. The food intake of ATXN2 KO mice is still a controversial matter: (i) in 2006, Kiehl and co-authors proposed that hyperphagia could be responsible for obesity, however no significant evidences that supported this theory were shown (*Kiehl et al., 2006*); (ii) in 2008, Lastres-Becker and team did not observe changes in weight gain in the KO mice compared to WT littermates (*Lastres-Becker et al., 2008b*); (iii) in 2012, Scoles reported hyperphagia in KO and heterozygous mice even though these results were not shown (*Scoles et al., 2012*). These discrepancies can be a consequence of the use of distinct diets with different percentages of fat. Interestingly, we observed that ATXN2 KO mice show lower levels of AgRP mRNA, and a tendency for NPY mRNA decrease and POMC increase. These neuropeptides changes are in accordance with the decreased food intake observed in ATXN2 knockout mice. The hypothalamic ataxin-2 overexpression in the KO mice promotes a trend to counteract this pattern of expression, however the reduced number of animals used, does not allowed statistical significance. Altogether these observations are not in favour of the theory proposed previously, in which the obesity of ATXN2 KO was caused by hyperphagia (*Kiehl et al., 2006; Scoles et al., 2012*). Moreover, these results suggest that body weight gain could be secondary to other metabolic impairments.

In order to investigate the cause of obesity in ATXN2 KO mice, we evaluate if a decrease in activity could explain body weight increase. Previous studies reported that ATXN2 KO mice are hyperactive (*Kiehl et al., 2006; Lastres-Becker et al., 2008b; Huynh et al., 2009*). Accordingly, we also observed that ATXN2 KO mice have higher motor activity. Moreover, this increase in activity was mostly observed in diurnal phase. In fact, ATXN2 KO mice showed attenuated differences between diurnal and nocturnal activity patterns, inactive and active phases

respectively. This might suggest that ATXN2 KO have circadian rhythm dysfunction. In fact, ATXN2 KO mice show lower levels of Bmal1 and CLOCK mRNA expression, which are rescued upon ataxin-2 hypothalamic overexpression. Bmal1 and CLOCK are core clock genes that initiate the translational feedback loop required for the 24h keeping. Alterations in these genes are directly linked to insulin resistance and obesity (*Jeong et al., 2015; Liu et al., 2016; Ribas-Latre and Eckel-Mahan, 2016*). These results are in accordance with studies in *Drosophila* where the authors show that ataxin-2 ortholog in *Drosophila* is responsible for the regulation of the translation of the clock gene, PERIOD (PER) (*Lim et al., 2011; Lim and Allada, 2013; Zhang et al., 2013*). Furthermore, ataxin-2 downregulation in *Drosophila* results in a long-period rhythm of constant dark, whereas the re-establishment of ataxin-2 levels restored the normal circadian rhythm (*Lim and Allada, 2013*). A recent study with ATXN2 KO also reports the decrease of *Rora* mRNA, a transcription factor implicated in circadian rhythm regulation (*Halbach et al., 2016*).

Alterations in circadian rhythm were already related to several metabolic disturbances and (*Eckel-Mahan and Sassone-Corsi, 2013; Challet, 2015; Coomans et al., 2015*). HFD feeding can increase daytime activity and the concomitant feeding during the light/inactive phase. Daytime feeding in mice or general feeding in a metabolic resting phase, such as shift workers in human studies, promotes several metabolic alterations like obesity and insulin resistance (*Kohsaka et al., 2007; Hatori et al., 2012; Sherman et al., 2012; Prasai et al., 2013; Chaix et al., 2014; Mukherji et al., 2015; Opperhuizen et al., 2016*). Our hypothesis is that since ATXN2 KO mice do not change their behaviour between the active and inactive phase, prolonguing their feeding window. Feeding patterns are also timekeepers, since depend on circadian rhythms that dictate the light/dark - inactive/active period. So these mice are not hyperphagic, however eat at the “wrong” metabolic period and this feeding pattern disturbs metabolic pathways, thus promoting obesity and insulin resistance. The results obtained in our study are coherent with an important role of ataxin-2 in the regulation of circadian rhythm. The suprachiasmatic nucleus of the hypothalamus (SCN) is the hypothalamic nuclei responsible for the regulation of the circadian rhythm (*Moore and Eichler, 1972; Stephan and Zucker, 1972*). Despite the fact that we performed the ataxin-2 overexpression in the ARH, the ability of ataxin-2 to modulate Bmal1 and CLOCK expression could be the cause for the improvement in insulin sensitivity observed.

Insulin resistance is one of the major consequences of obesity and underlies type 2 diabetes. ATXN2 KO mice are insulin resistant, probably due to the defective processing of the InsR (insulin receptor) (*Lastres-Becker et al., 2008b*). In the present study we observed that

hypothalamic ataxin-2 increases insulin sensitivity and rescues insulin response. ATXN2 KO mice have an impaired response to insulin, as well as a decreased mRNA expression of *InsR* and *IRS1* in the hypothalamus, suggesting alterations in insulin signalling (*Boucher et al., 2016*). The ataxin-2 ARH overexpression promoted a trend to improve insulin response and promoted an increase in *InsR* and *IRS1* mRNA levels in the hypothalamus. Ataxin-2 has been proposed to participate in the endocytosis of tyrosine kinase receptors such as InsR (*Nonis et al., 2008; Fittschen et al., 2015*). In the KO mice, the absence of ataxin-2 might compromise this process leading to defective insulin signalling and consequent resistance. The restoration of ataxin-2 in the hypothalamus might improve this mechanism and rescue insulin response. Moreover, ataxin-2 has an active role in RNA metabolism; it can bind to 3'UTR of mRNA in order to promote its stability and translation (*Yokoshi et al., 2014*). The increase of mRNA expression of *InsR* and *IRS1* could also be a result from the effect of ataxin-2 in the RNA processing steps. For all this, the improvement of insulin sensitivity upon ataxin-2 re-establishment in the hypothalamus of ATXN2 KO mice might result from cumulative beneficial effects, like the restoration of circadian genes expression, and the restoration of factors in the insulin signalling pathway.

In respect of insulin signalling pathways, the over-activation of mTOR and its substrate RPS6K lead to insulin desensitization (*Yoon and Choi, 2016; Boucher et al., 2016*). As described, ataxin-2 absence promotes the hyperactivation of the mTOR substrate, RPS6K (*Lastres-Becker et al., 2016*). On other hand, yeast ortholog of ataxin-2 Pbp1, can sequester TORC1 to stress granules and inhibit this pathway in nutrient-rich conditions (*Takahara and Maeda, 2012; Buchan et al., 2012; DeMille et al., 2015*). Using an *in vitro* approach we demonstrated that ataxin-2 levels are decreased upon palmitate stimulation. Palmitate is commonly used as an insulin resistance *in vitro* model. Ataxin-2 overexpression was able to revert the overactivation of the mTOR pathway mediators induced by palmitate, supporting the role of ataxin-2 in the hypothalamic regulation of this pathway. The prevention of the overactivation of the mTOR pathway observed in ATXN2 KO mice could also be related with the ability of hypothalamic ataxin-2 to promote insulin sensitivity.

Insulin signalling in the hypothalamus is responsible for the suppression of hepatic glucose production and the regulation of adipose tissue lipolysis (*Obici et al., 2002; Scherer et al., 2011).* The loss of the hypothalamic autonomic regulation by insulin, promotes the lipolysis of WAT and the release of free fatty acids that can accumulate in the liver. The lipid accumulation in the liver compromises glucose metabolism, promoting hyperglycaemia, which will further exacerbate the overall insulin resistance (*Samuel and Shulman, 2012; Bears and Perry, 2015;*

*Gancheva and Roden, 201*6). Insulin resistance and the overall metabolic dysfunctions work therefore in a "vicious cycle". We observed that ATXN2 KO mice have lipid accumulation in the liver and the hypothalamic overexpression of ataxin-2 is able to decrease this event. Moreover, ataxin-2 hypothalamic overexpression in the KO mice also promoted a decrease in adipocyte size of the WAT. Nevertheless, we did not observe a relevant effect of ataxin-2 re-establishment on BAT, which have a "whiter" aspect than in WT littermates. The increase in insulin sensitivity promoted by ARH ataxin-2 restoration in the KO mice might explain the improvement in periphery metabolic organs. The fact that ataxin-2 overexpression in the ARH could not influence BAT physiology might result from the fact that areas beyond the ARH have a more direct impact in the autonomic control of BAT.

Altogether, the present study shows for the first time that ataxin-2 plays a role in the hypothalamus, especially in insulin signalling. We also demonstrate that ATXN2 KO mice displays disruption at circadian clock genes, which might be responsible for their obese and insulin resistant phenotypes. Moreover, hypothalamic ataxin-2 overexpression restores clock genes expression and improve insulin sensitivity in ataxin-2 knockout mice. These observations may suggest a relevant potential of ataxin-2 as a therapeutic target for disorders with impaired insulin response.

CHAPTER 7

Concluding Remarks

7. Concluding remarks

In the present work, we unravel the role of ataxin-2 in hypothalamic-regulated functions, namely body weight, food intake, insulin sensitivity and the autonomic regulation of liver and adipose tissue. Ataxin-2 is of particular interest in the context of obesity given the fact that the gene encoding for ataxin-2 is part of the *ATXN2/SH2B3* trait. Different studies show that polymorphisms in this trait increase predisposition for obesity and obesity-related diseases (*Li et al., 2004; Chagnon et al., 2004; Dong et al., 2005; Auburger et al., 2014*). Moreover, ataxin-2 has been gaining relevant interest as a metabolic mediator participating in processes such body weight and fat distribution, lipid metabolism regulation, insulin signalling and metabolic pathways modifier (*Carmo-Silva et al., 2017*).

With the present work we demonstrate, for the first time, that HFD-induced obesity decreases ataxin-2 expression in the hypothalamus, which might suggest that ataxin-2 is as a possible metabolic sensor. Silencing ataxin-2 in the arcuate nucleus of the hypothalamus had a negative impact on body homeostasis, promoting obesity, hyperphagia, insulin resistance, hepatosteatosis and hypothalamic inflammation. We show for the first time that ataxin-2 silencing mimics metabolic deregulation promoted by over-nutrition. These results support a role for ataxin-2 in hypothalamic physiology.

In chapter 4 of the presented work, given the decrease in hypothalamic ataxin-2 upon HFD, we re-established of ataxin-2 in the hypothalamus. The hypothalamic re-establishment of ataxin-2 prevented body weight gain and other metabolic alterations such as insulin resistance, hepatosteatosis, whitening of the brown adipose tissue and hypothalamic dysfunction. We still do not know if the improvement in metabolic condition is due to the weight gain prevention or the increased insulin sensitivity. However, these results support ataxin-2 as a promising target for anti-obesity strategies.

We used ATXN2 KO mice to further investigate the role of ataxin-2 in the hypothalamus. These ATXN2 KO mice present a phenotype of severe metabolic compromise: obesity, insulin resistance and infertile, with hepatosteatosis and dyslipidaemia (*Kiehl et al., 2006; Lastres-Becker et al., 2008b*). Moreover, independent studies further demonstrated in these mice, altered branched chain amino-acid metabolism and compromised metabolic pathways, namely overactivation of mTOR signalling (*Meierhofer et al., 2016; Lastres-Becker et al., 2016*).

We demonstrate for the first time that ATXN2 KO mice have alterations in circadian clock genes expression and circadian rhythm locomotor activity. A dysfunction in the balance of

clock genes expression is tightly connected to obesity and insulin resistance (*Shi et al., 2013; Coomans et al., 2015; Ribas-Latre and Eckel-Mahan, 2016*), what can in fact explain the phenotype of ATXN2 KO mice. Furthermore, ataxin-2 hypothalamic restoration in ATXN2 KO mice can revert the altered expression of clock genes. This observation suggests a new role for ataxin-2 in the hypothalamus, as a circadian rhythm regulator. Moreover, it can support the observation of increased insulin sensitivity in ATXN2 KO mice upon ataxin-2 hypothalamic overexpression. Additional studies are needed to better understand the role of ataxin-2 in the circadian rhythm and the effect on overall metabolism.

Ataxin-2 role on insulin sensitivity might be a result from different effects in hypothalamus. In one hand, ataxin-2 hypothalamic rescue is able to restore circadian genes expression, and the overexpression of ataxin-2 in the hypothalamus of ATXN2 KO mice also rescues InsR and IRS1 expression. The improvement of insulin cascade mediators can directly ammeliorate insulin sensitivity (*Boucher et al., 2014*). Moreover, our *in vitro* studies allowed us to show that the increase or decrease of ataxin-2 in hypothalamic neurons changes the activation of the mTOR pathway. And mTOR overactivation is directly correlated with insulin resistance. Therefore, ataxin-2 ability to decrease the activation mTOR can also contribute to the beneficial effects of ataxin-2 on insulin sensitization. Overall these results suggest that hypothalamic ataxin-2 is an insulin sensitivity modulator and a promising therapeutic target for type 2 diabetes and metabolic dysfunction.

In summary, the major conclusions of this work are:

1) Hypothalamic ataxin-2 functions as a metabolic sensor, since HFD decreases its expression;
2) Hypothalamic ataxin-2 silencing promotes obesity and metabolic dysfunction, hence ataxin-2 overexpression in the hypothalamus is a potent anti-obesity strategy;
3) Ataxin-2 KO mice present alterations in the circadian behaviour that can be related to the overall metabolic dysfunction presented by these mice;
4) Ataxin-2 has a determinant role in insulin signalling in the hypothalamus;
5) Specific ataxin-2 overexpression in the hypothalamus improves insulin sensitivity and the overall metabolic homeostasis.

CHAPTER 8

References

8. References

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