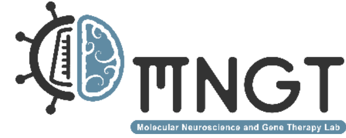

UNIVERSIDADE DO ALGARVE

Faculdade de Ciências e Tecnologia

Targeting autophagy as a therapeutic strategy for spinocerebellar ataxia type 2

Ana Rita Tiago Rosa

Dissertação para obtenção do grau de Mestre em Ciências Farmacêuticas

Trabalho efetuado sob a orientação de:

Professor Doutor Clévio Nóbrega, ABC Research Institute

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Declaração de Autoria de Trabalho:

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“How you start is important, but it is how you finish that counts. In the race for success, speed is less important than stamina. The sticker outlasts the sprinter.”

Bertie Charles Forbes

Agradecimentos

Este percurso foi sem dúvida complicado e desafiante! Chegar ao fim desta etapa deixa-me bastante feliz, uma vez que, apesar de todos os obstáculos que têm surgido e que me têm impedido de avançar como gostaria, significa que concluí mais uma importante etapa da minha vida. Assim gostaria de agradecer a todos os que, de alguma forma, me ajudaram e apoiaram durante este percurso que irá culminar com a obtenção do grau de mestre em ciências farmacêuticas.

Ao meu orientador, Professor Doutor Clévio Nóbrega, agradeço pelo privilégio e a incrível oportunidade que me deu por ter aceite a minha orientação e poder assim integrar o seu grupo de investigação, Molecular Neuroscience and Gene Therapy, no Algarve Biomedical Center – Research institute. Gostaria ainda de agradecer por toda a paciência, ajuda, motivação, disponibilidade e por toda a confiança que depositou em mim ao longo deste percurso.

A todo o grupo de investigação, mas em especial à Adriana, obrigada pela amizade, motivação, ajuda, esclarecimento de dúvidas e apoio ao longo deste percurso.

Às minhas orientadoras de estágio, Drª. Paula Campos (estágio em farmácia hospitalar) e Drª. Ângela Ferreira (estágio em farmácia comunitária) e restantes equipas, um obrigada por toda a disponibilidade, ajuda, apoio e transmissão de conhecimentos durante os estágios.

A todos os professores do curso Mestrado Integrado em Ciências Farmacêuticas obrigada por todos os ensinamentos transmitidos e em especial, um obrigado à professora Isabel Ramalhinho, que me acompanhou nesta fase final do meu percurso.

A todos os meus amigos, cujo apoio e amizade estiveram sempre presentes e que não me deixaram ir abaixo nos diversos obstáculos com que me deparei neste trajeto.

A toda a minha família que foi essencial, mas em especial aos meus pais e à minha irmã, o meu enorme obrigada por toda a motivação, incentivo, ajuda, paciência, apoio incondicional durante este meu percurso, que se tornou mais longo, mais difícil e desafiante do que eu previa. Muito obrigada por me darem força e acreditarem sempre em mim!

Resumo

A ataxia espinocerebelosa tipo 2 (SCA2) é uma doença hereditária autossómica dominante que, em conjunto com outras oito doenças neurodegenerativas, forma um grupo de doenças, denominado por doenças de poliglutaminas. Estas são causadas por uma expansão anormal do trinucleótido CAG (citosina-adenina-guanina), que leva à formação de uma proteína anormal, o que afeta a homeostase celular.

A SCA2 foi descrita pela primeira vez em 1971 e é causada por um aumento de mais de 31 repetições CAG no gene *ATXN2*. Este codifica uma proteína ataxina-2 anormal que não é eficientemente degradada, acumulando-se e levando à formação de agregados tóxicos, presentes principalmente no citoplasma. Esses agregados levam a diferentes mecanismos de patogénese, como a disfunção da autofagia, que é responsável pela degradação e reciclagem de proteínas.

Ainda não existe cura para a SCA2, porém atualmente existem várias estratégias terapêuticas em estudo, como a redução do número de agregados através da ativação da autofagia pela cordicepina.

Assim, o presente estudo teve como objetivo estudar a autofagia na SCA2 e perceber se a indução desta pode vir a ser uma futura estratégia terapêutica.

Os resultados demostraram uma disfunção da via autofágica em células Neuro-2A transfectadas com formas mutantes de ataxina-2, pela verificação de um aumento dos níveis de SQSTM1 e de uma diminuição dos níveis de LC3B-II, e no tecido cerebral *post-mortem* de pacientes com SCA2, pela observação de um aumento dos agregados de ataxina-2 e de uma acumulação anormal de marcadores autofágicos. Além disso, demonstraram ainda que a administração de cordicepina leva à diminuição da perda neuronal e à redução da agregação da proteína ataxina-2.

Assim e apesar de serem necessários mais estudos complementares este estudo demonstrou que a regulação positiva da autofagia pode ser uma futura estratégia terapêutica para o tratamento da doença SCA2.

Palavras-Chave: Doenças de poliglutaminas, Ataxia espinocerebelosa tipo 2, Ataxina-2, Autofagia, Cordicepina.

Abstract

Spinocerebellar ataxia type 2 (SCA2) is an autosomal dominant inherited disease, which together with eight other neurodegenerative diseases, forms a group of diseases, called polyglutamine diseases. These are caused by an abnormal expansion of the CAG (cytosine-adenine-guanine trinucleotide), which leads to the formation of an abnormal protein, that affects cellular homeostasis.

SCA2 was first described in 1971 and is caused by an increase of more than 31 CAG repeats in the *ATXN2* gene. This encodes for an abnormal ataxin-2 protein that is not efficiently degraded, accumulating and leading to the formation of toxic aggregates, present mainly in the cytoplasm. These aggregates lead to different pathogenesis mechanisms, such as autophagy dysfunction, responsible for degradation and recycling of proteins.

There is still no cure for SCA2, but there are currently several therapeutic strategies under study, such as reducing the number of aggregates through the activation of autophagy by cordycepin.

Therefore, the present study aimed to study autophagy in SCA2 and to understand whether its induction can become a future therapeutic strategy.

The results demonstrated a dysfunction of the autophagic pathway in Neuro-2A cells transfected with mutant forms of ataxin-2, by verifying an increase in the levels of SQSTM1 and a decrease in the levels of LC3B-II, and in the post-mortem brain tissue of patients with SCA2, by observing an increase in ataxin-2 aggregates and an abnormal accumulation of autophagic markers. Furthermore, administration of cordycepin has been shown to lead to decreased neuronal loss and reduced aggregation of ataxin-2 protein.

Thus, and despite the need for further complementary studies, this study demonstrated that the upregulation of autophagy may be a future therapeutic strategy for the treatment of SCA2 disease.

Keywords: Polyglutamine diseases, Spinocerebellar ataxia type 2, Ataxin-2, Autophagy, Cordycepin.

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Abbreviations list

Ago1: Argonuate 1

AMP: Adenosine monophosphate

AMPK: Adenosine monophosphate activated kinase

***AR*:** Androgen receptor

ASOs: Antisense oligonucleotides

Atg 4, 5, 7, 9, 10, 12, 16: Autophagy-related protein 4, 5, 7, 9, 10, 12, 16

***ATN1*:** Atrofin-1

ATP2a2: ATPase Sarcoplasmic/Endoplasmic Reticulum $Ca2^{+}$ Transporting 2

***ATXN1, 2, 3, 6, 7, 17*:** Ataxin 1, 2, 3, 6, 7, 17

Atx2-Q22: Ataxin-2 with a polyQ segment with 22 glutamines

Atx2-Q58: Ataxin-2 with a polyQ segment with 58 glutamines

Atx2-Q104: Ataxin-2 with a polyQ segment with 104 glutamines

A2BP1: Ataxin-2-RNA-binding protein

Bcl2: B-cell lymphoma 2 protein)

BSA: Bovine serum albumin

***CACNA1A*:** α_1-voltage dependent calcium channel subunit

CAG: Cytosine-adenine-guanine trinucleotide

cAMP: Cyclic adenosine monophosphate

ChQ: Chloroquine

CNS: Central nervous system

CoMP: Cordycepin 5'-monophosphate

DNA: Deoxyribonucleic acid

DMEM: Dulbecco's modified Eagle's medium

DMSO: Dimethyl sulfoxide

DRPLA: Dentatorubral pallidoluysian atrophy

EGFP: Enhanced green fluorescent protein

EiF4F: Eukaryotic initiation factor 4F

ENT1/ENT2: Adenosine transporter

Epac: Exchange factor directly activated by cAMP

GCS: Granular cytoplasmatic staining

HD: Huntington's disease

hMSCs: Human mesenchymal stem cells

Hsc 70: Heat shock cognate protein of 70 kDa

Hsp 70: Heat shock protein 70

***HTT*:** Huntingtin

Inpp5a: Inositol Polyphosphate-5-Phosphatase A

IP: Intraperitoneal injection

IP_3: $Ins(1,4,5)P_3$

Itpr 1: Inositol (1, 4, 5) - triphosphate type 1 receptor

kDa: kiloDaltons

KFERQ: Lysine-phenylalanine-glutamic acid-arginine-glutamine like pentapeptide motif

LAMP2A: Lysosome-associated membrane receptor type 2A

LC3B-I, II: Microtubule-associated protein 1 light chain 3 beta type I, II

Lsm: Like Sm domain

LsmAD: Lsm associated domain

NaCl: Sodium chloride

NeuroEPO: Human recombinant erythropoietin

Neuro-2A: Mouse neuroblastoma line-derived cells

NNI: Neuronal nuclear inclusions

ME31B: Deadbox helicase Me31B

mRNA: Messenger RNA

mTOR: Mammalian target of rapamycin

PABP: Polyadenylate-binding protein

PAB6: Polyadenylate-binding protein 6

PAM2: PABPC1- interactingmotif-2

PBS: Phosphate buffer saline

PE: Phosphatidylethanolamine

PEI: Polyethylenimine

PIP_2: Phosphatidylinositol 4,5-bisphosphate

PI3K: Phosphatidylinositol 3 kinase

PolyQ: Polyglutamine

RBM9: RNA-binding motif protein 9

RBPMS: RNA-binding protein with multiple splicing

RIPA-buffer: Radioimmunoprecipitation assay buffer

RNA: Ribonucleic acid

RNAi: RNA interference

rora: RAR Related Orphan Receptor A

RPS6K: Ribosomal protein S6

SBMA: Spinal and bulbar muscular atrophy

SCAs: Spinocerebellar ataxias

SCA1, 2, 3, 6, 7, 17: Spinocerebellar ataxia type 1, 2, 3, 6, 7, 17

ShRNA: Short hairpin RNA

SiRNA: Synthetic small interfering RNA

SQSTM1: Sequestosome protein 1

***TBP*:** TATA box binding protein

Ulk1: Unc-51-like kinase

UPS: Ubiquitin-proteosome system

WB: Western blot

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

1. Introduction

1.1. Polyglutamine diseases

In 1991, a neurological disorder caused by unstable repeats was first described, when the causative mutations of fragile X syndrome and spinal and bulbar muscular atrophy (SBMA) were identified (Gatchel and Zoghbi, 2005). SBMA belongs to the polyglutamine (polyQ) diseases group, which are caused by an abnormal expansion of unstable nucleotides, since they are characterized by an expansion of CAG repeats (cytosine-adenine-guanine trinucleotide) in the coding region of affected genes (Giuffrida et al., 1999, Riley and Orr, 2006).

The polyQ diseases are a group of nine untreatable and hereditary neurodegenerative disorders, that include six spinocerebellar ataxias (type 1, 2, 3, 6, 7 and 17), Huntington's disease (HD), dentatorubral pallidoluysian atrophy (DRPLA) and SBMA (La Spada and Taylor, 2003, Orr and Zoghbi, 2007, Riley and Orr, 2006). This group of diseases are all autosomal dominantly inherited, with one exception, the SBMA, that is a disease linked to X-chromosome (Fan et al., 2014).

All the diseases mentioned above share several pathological features, like anticipation (symptoms are worse from generation to generation), intracellular multiprotein inclusions in neuronal tissue (although in different areas), disease onset at middle age, and death after 15-20 years with progressive aggravation of the symptoms. Moreover, the longer the expansion of CAG repeats, the earlier the symptoms appear and higher the number of protein aggregates observed in specific areas of the brain. These common features may suggest that protein with an expanded polyQ tract, cause a similar toxic effect regardless of the diverse phenotype. Despite this common characteristics and all belonging to the class of polyQ diseases, they are caused by mutations in different genes, affect different areas of the nervous system and have different clinical manifestations (Table I) (Fan et al., 2014, Zoghbi and Orr, 2000, Cummings and Zoghbi, 2000, Durr, 2010, Schöls et al., 2004, Gatchel and Zoghbi, 2005, Williams and Paulson, 2008, Stoyas and La Spada, 2018).

Table I

Comparison of molecular characteristics and clinical symptoms between polyQ diseases

Diseases	Gene	Protein	CAG repeats		Clinical Symptoms
			Normal	Disease	
DRPLA	*ATN1*	Atrophin-1	7-35	49-88	Myoclonic epilepsy, cognitive deficits, choreoathetosis, ataxia
HD	*HTT*	Huntingtin	6-35	39-250	Chorea, cognitive deficits, psychiatric disturbances
SBMA	*AR*	Androgen receptor	5-34	37-70	Muscular atrophy, weakness, bulbar palsy
SCA1	*ATXN1*	Ataxin-1	6-44	>39	Ataxia, pyramidal signs, muscular atrophy, bulbar palsy
SAC2	*ATXN2*	Ataxin-2	13-31	>31	Ataxia, neuropathy, slow eye movement
SCA3	*ATXN3*	Ataxin-3	12-40	55-84	Ataxic, parkinsonism, spasticity, bulging eye, fasciculations
SCA6	*CACNA1A*	CACNA1A	4-18	19-33	Ataxia
SCA7	*ATXN7*	Ataxin-7	4-35	37-306	Ataxia, retinal degeneration
SCA17	*TBP*	TATA box binding protein	25-48	43-66	Ataxia, cognitive deficits, dystonia, parkinsonism

AR: Androgen receptor; *ATN1*: atrofin-1; *ATXN1, 2, 3, 6, 7, 17*: Ataxin-1, 2, 3, 6, 7, 17; *CACNA1A*: α_{1A}-voltage-dependent calcium channel subunit; *HTT*: Huntingtin; *TBP*:

TATA box binding protein; SCA1, 2, 3, 6, 7, 17: Spinocerebellar ataxia type 1, 2, 3, 6, 7, 17. [Adapted from (Stoyas and La Spada, 2018, Banno et al., 2009)].

In polyQ diseases the over repetition of the CAG trinucleotide is translated and form a large polyQ tract, which cannot be efficiently degraded. Consequently, the accumulation of polyQ proteins leads to alterations in cellular homeostasis (Bennett et al., 2007, Chafekar and Duennwald, 2012, Fan et al., 2014). Thus, aggregated polyQ proteins contributes to degeneration and dysfunction in specific neuronal subpopulations in the cerebellum, brainstem, and spinal tract (Zoghbi and Orr, 1999, Gatchel and Zoghbi, 2005).

1.2. Spinocerebellar ataxia type 2

Spinocerebellar ataxias (SCAs) are a group of hereditary neurological diseases, which mostly affects the central nervous system (CNS) (Schöls et al., 2004, Rüb et al., 2013). Until now, there are more than forty genetically distinct subtypes of SCAs and are identified with numbers. These numbers represent the chronological order in which the disease locus or gene was identified. (Synofzik and Németh, 2018, Zanni and Bertini, 2018).

Patients with these progressive neurodegenerative diseases may present different manifestations as cerebellar ataxia, which results in unsteady gait, clumsiness, and dysarthria. This group of diseases, sometimes, is also associated with other visual impairments like nystagmus, ophthalmoplegia and slow saccadic eye movements and neurological signs such as pyramidal or extrapyramidal signs, and cognitive impairment (Harding, 1983).

Normally, the onset of ataxia is in mid-adulthood, but can occur in childhood (130 to 200 CAG repeats) and old age too (Durr, 2010). There is still no effective neuroprotective treatment for this group of diseases and patients with SCAs have a shorter life span and worse quality of life (Fan et al., 2014).

Spinocerebellar ataxia type 2 (SCA2) was not always called this way. In 1971, it was known as "Wadia-Swami-type ataxia", when it was first described in nine patient families by two Indian researchers (Wadia and Swami), who called attention to early

saccadic velocity of the eyes. Later, in 1996, it was renamed "Spinocerebellar Ataxia Type 2", with the discovery of the disease locus on chromosome 12. SCA2, together with SCA6, is the second most common autosomal dominantly inherited subtype of ataxia in the world (15% of total cases) and the third most common within the group of polyQ diseases, after HD and SCA3 (Lastres-Becker et al., 2008, Magaña et al., 2013, Alves-Cruzeiro et al., 2016, Wadia and Swami, 1971, Sinha et al., 2004, Gispert et al., 1993).

The global prevalence of SCA2 is around 3 in 100.000 individuals but there is a regional variation. SCA2 is mostly detected in Holguín (Cuba), where, due a founder effect, frequency of cases is 40 per 100.000 for Spanish ancestry individuals. It is also detected in Brazil, Germany, Italy, Australia, United Kingdom, Spain and is the ataxia form most prevalent in Mexico and eastern India (Sinha et al., 2004, Alonso et al., 2007, Velásquez-Pérez and Ramírez-Crescencio, 2014, Velázquez-Pérez et al., 2011b, Leggo et al., 1997, Giunti et al., 1998, Pujana et al., 1999, Infante et al., 2005, Alves-Cruzeiro et al., 2016, Ruano et al., 2014, Orozco Diaz et al., 1990).

1.2.1. Clinical, physiological, and neuropathological features

Beyond progressive gait ataxia, that is a hallmark of any SCA, the clinical features of SCA2 also include dysarthria accompanied by leg cramps, postural tremors, decreased muscle tone, and decreased tendon reflexes, sleep disturbances and oculomotor dysfunctions. Symptoms such as depression, insomnia, cognitive disorders, lack of memory and suicide attempts may also be observed (Pulst, 2015, Bürk et al., 1996, Lastres-Becker et al., 2008, Alves-Cruzeiro et al., 2016, Geschwind et al., 1997, Ashizawa et al., 2013, Gwinn-Hardy et al., 2000). These symptoms start in the fourth decade of life and are progressive worst until the death of the patient, normally 10 years after the onset of symptoms (Matos et al., 2017, Lastres-Becker et al., 2008, Klockgether and Evert, 1998, Alves-Cruzeiro et al., 2016).

The main affected area is the cerebellum, especially the Purkinje cells, although recently other brain regions were also implicated in the disease, for example the *substantia nigra*, striatum or the neocortex (Matos et al., 2017).

The disease quickly evolves into a multisystemic neurodegeneration, which affect the brainstem (saccadic velocity), the spinal cord (deep tendon reflexes, decreased

vibration sense), the midbrain (sometimes manifested as parkinsonism) and the muscles (cramps) (Maschke et al., 2005, Rosa et al., 2006, Auburger, 2012).

Over time, symptoms such as immobility together with distal amyotrophy, dysphagia, ophthalmoplegia, incontinence and mental deficiencies are observed. The final stages of SCA2 are characterized by severe autonomic problems, like vasomotor, cardiac, gastrointestinal, urinary, exocrine gland dysfunction, and finally ends with respiratory failure, due to motoneuron degeneration (Bürk et al., 1996, Abele et al., 1997, Orr and Zoghbi, 2000, Auburger, 2012, Sánchez-Cruz et al., 2001).

Briefly and compared with other SCAs, SCA2 has four main features besides cerebellar ataxia: early saccadic slowing, early hyporeflexia, severe tremor (of postural or action type), and early myoclonus. The other symptoms may be absent, mainly in patients with small polyglutamine expansions (Auburger, 2012).

The neuropathological features of SCA2 are degeneration and loss of cerebellar Purkinje neurons followed by retrograde degeneration of inferior olive neurons. Also loss of dopaminergic neurons is observed in the *substantia nigra*, an overlapping feature of SCA2 and SCA3 (Koeppen, 2018a, Estrada et al., 1999, Koeppen, 2018b, Scherzed et al., 2012, Seidel et al., 2012, Chen et al., 2012, Adachi et al., 2015).

In vivo brain, magnetic resonance imaging showed early atrophy of the cerebellum, midbrain and brainstem in the pons/olive area (particularly the middle cerebellar peduncle) and general ventricular enlargement (Bürk et al., 1996, Giuffrida et al., 1999, Brenneis et al., 2006, Brenneis et al., 2003). In a pontocerebellar distribution is detected by positron emission tomography an earliest glucose hypometabolism, loss of dopamine transporters and nigrostriatal atrophy (Inagaki et al., 2005, Boesch et al., 2004, Wüllner et al., 2005). Proton magnetic resonance spectroscopic imaging, suggesting an alteration of glycolysis and mitochondrial function due to the high values of lactate (Boesch et al., 2001).

In *post-mortem* pathoanatomical studies, were made a diagnosis of olivo-ponto-cerebellar atrophy and macroscopic inspection shows marked atrophy of the cerebellum, pons, frontal lobe, medulla oblongata, thalamus, cranial nerves, and pallor of the midbrain *substantia nigra* (Estrada et al., 1999, Gierga et al., 2005, Rüb et al., 2005, Rüb et al., 2007).

In the spinal cord is observed a decrease in the number and size of motor neurons (at the cervical level) and Clarke's column neurons (in globus pallidus, thalamus, subthalamus), and a concomitant increase of astrocytes and microglia (in periaqueductal region) (Huynh et al., 1999).

At the cellular level, there is a neuronal loss in the granular layer of the cerebellum, brain stem and *substantia nigra,* which can explain the parkinsonism symptoms in SCA2. There is also a reduction in the number and arborization of Purkinje cells (Pulst, 2015, Estrada et al., 1999, Rüb et al., 2013).

Protein aggregates are a hallmark of neurodegeneration in most polyQ diseases. Among all spinocerebellar ataxias, SCA2 has the lowest number of neuronal nuclear inclusion bodies, being detected only in pontine neurons and rarely in the inferior olive. Thus, it appears that the mutated ataxin-2 protein leads to the formation of aggregates that are mostly in the cytoplasm, thus being less recruited for neuronal nuclear inclusions (only 1-2%) than other polyQ proteins (Uchihara et al., 2001, Pang et al., 2002, Huynh et al., 2000).

1.2.2. Diagnosis of SCA2 disease

The clinical features of SCA2 overlap with other SCAs, so an accurate diagnosis must be made through the identification of the genetic mutation that causes the disease (Lastres-Becker et al., 2008). However, a detailed family history, physical examination and checking for certain symptoms can be used to complete the diagnosis or even for a diagnosis if genetic testing cannot be performed (Auburger, 2012).

1.2.3. The SCA2 gene

There are two different groups of SCAs: those caused by repeat expansions mutations, like SCA2 and those caused by non-repeat mutations. Both types share a common feature, anticipation (symptoms are worse from generation to generation) (Klockgether et al., 2019).

In SCA2 disease, the *ATXN2* gene, present in exon 1 (coding region), has a polyglutamine-coding region and under normal conditions it has between 13 and 31 CAG repeats. In SCA2 patients an abnormal expansion of the CAG repeats is observed (above

31 CAGs, and that can go up to 200), which lead to a neurodegenerative disorder, SCA2 (Figure 1) (Scoles and Pulst, 2018, Magaña et al., 2013). Like in other polyQ diseases, there is an inverse proportionality between the age of onset of the disease and the number of CAG repeats, i.e., the greater the number of trinucleotides repeated, the earlier the onset of symptoms of this disease occurs (Maciel et al., 1995, Maruyama et al., 1995, Globas et al., 2008).

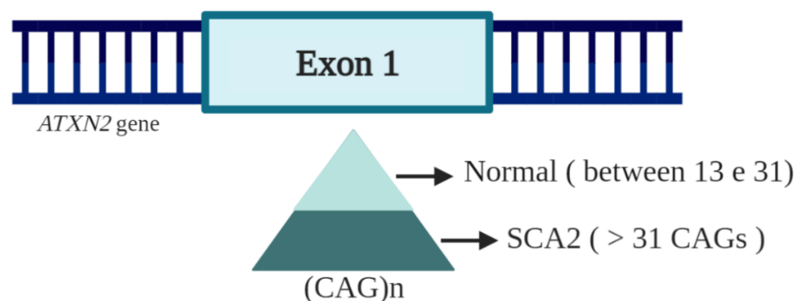

Figure 1: Variation in the number of CAG repeats in the ATXN2 gene. In exon 1 of the *ATXN2* gene there is a polyQ-coding tract and the number of CAG repeats determines whether there is disease or not. Clinical manifestations of SCA2 are only observed if the number of CAG repeats is greater than 31 (and up to 200) [Adapted from (Scoles and Pulst, 2018, Magaña et al., 2013)].

The *ATXN2* gene is located on chromosome 12q24.1, is composed of 25 exons and encodes for ataxin-2 protein, which normally has 22 glutamines and molecular mass of approximately 140 kilodaltons (kDa) (Gispert et al., 1993, Albrecht et al., 2004, Scoles et al., 2012).

1.2.4. Ataxin-2 protein

The ataxin-2 protein is a basic protein (isoelectric point, 9.6), its normal form has 1312 amino acids and in humans it is mostly expressed in the CNS, especially in the *substantia nigra*, brainstem, spinal cord and Purkinje cells in the cerebellum (van de Loo et al., 2009).

Ataxin-2 protein can usually be found in the cytoplasm, however SCA2 protein inclusions are observed both in the nucleus (less quantity) and in the cytoplasm (Lastres-Becker et al., 2008, Koyano et al., 1999, Yokoshi et al., 2014).

Structurally, ataxin-2 protein is composed by a N-terminal and C-terminal, both rich in prolines, a glutamine-rich domain, two globular domains (acid region) named Like Sm domain (Lsm), which is involved in RNA (ribonucleic acid) processing and Lsm-associated domain (LsmAD), that contains an endoplasmic reticulum exit signal and a clathrin-mediated trans-Golgi signal (Figure 2). The structure of the ataxin-2 protein also has a region with a PAM2 motif (PABPC1-interactingmotif-2) towards C-terminal end, which is responsible for the interaction with PABP (polyadenylate-binding protein) (Figure 2) (Albrecht et al., 2004, Lastres-Becker et al., 2008, Satterfield and Pallanck, 2006).

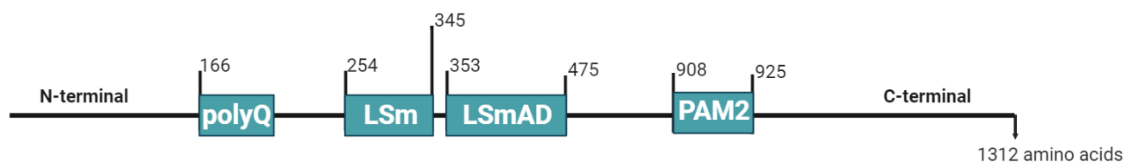

Figure 2: Structure of ataxin-2 protein. The ataxin-2 protein contains 1312 amino acids, has a molecular mass of approximately 140 kDa and, structurally, is composed by a polyQ tract at the N-terminal, two globular domains, the Lsm domain (amino acids 254-345) and LsmAD domain (amino acids 353-475) and a PAM2 motif (amino acids 908-925) towards C-terminal end [Adapted from (Magaña et al., 2013)].

1.2.5. Ataxin-2 functions

The functions of the ataxin-2 protein still need to be fully clarified, but a greater knowledge of the normal function of this protein may help to better understand the SCA2 disease and thus leading to a new discovered of an efficient treatment (Magaña et al., 2013).

The ataxin-2 protein has several functions, including the regulation of several RNA processing pathways, endocytosis, modulation of calcium signalling pathways and metabolism modulation (Magaña et al., 2013, Carmo-Silva et al., 2017).

1.2.5.1. Posttranscriptional regulation

The ataxin-2 protein seems to be involved in post-transcriptional regulation, since when comparing the domains of this protein with others already characterized, the Lsm domain of ataxin-2 is similar to domains of other proteins involved in RNA posttranscriptional regulation (Albrecht et al., 2004, van de Loo et al., 2009, Meunier et al., 2002).

Furthermore, studies have shown that the PAM2 domain of ataxin-2 protein interacts with the PAB6 (polyadenylate-binding protein 6) domain of the PABP (Figure 3), which is responsible for stability, translation regulation and alternative splicing of messenger RNA (mRNA) (Ralser, Albrecht, et al., 2005; Tharun, 2009). Studies have also revealed that the C-terminal of ataxin-2 protein interacts with ataxin-2-RNA-binding protein (A2BP1), responsible for mRNA splicing regulation. Ataxin-2 also interacts with RNA-biding motif protein 9 (RBM9) and RNA-binding protein with multiple splicing (RBPMS), both with the function of RNA splicing (Bravo et al., 2005, Tsien et al., 1996).

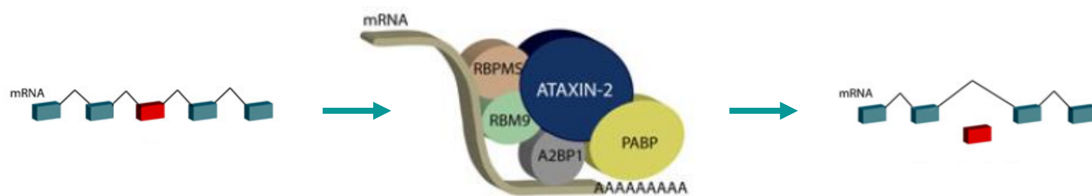

Figure 3: Ataxin-2 protein and posttranscriptional regulation. Ataxin-2 protein is involved in post-transcriptional regulation, specifically in the alternative splicing process, through interaction with PABP, A2BP1, RBM9 and RBPMS proteins [Adapted from (Magaña et al., 2013)].

Thus, and according to these studies, it seems that one of the functions of ataxin-2 protein is post-transcriptional regulation through its interaction with PABP, A2BP1, RBM9 and RBPMS proteins (Tharun, 2009, Ralser et al., 2005a, Bravo et al., 2005, Tsien et al., 1996).

1.2.5.2. Translational regulation

The ataxin-2 protein seems to be involved in the promotion of translation, since studies demonstrate that through Lsm/LsmAD and PAM2 domain independently, ataxin-2 protein interacts with polyribosomes through PABP (Figure 4). Furthermore, studies shown that ataxin-2 protein (that has a LsmAD domain with an endoplasmic reticulum export signal and a clathrin-mediated signal sequence) is in the rough endoplasmic reticulum bound to polyribosomes of neuronal cells (Albrecht et al., 2004, van de Loo et al., 2009).

In addition, a study focused on SCA3 disease showed that mutant ataxin-3 leads to an abnormal decrease in ataxin-2 levels causing an increase in the translation of mutant ataxin-3 and other proteins, which aggravates SCA3 disease. Beyond that, through the normalization of mutant ataxin-2 levels, a reduction in mutant ataxin-3 levels was verified and, consequently, the improvement of SCA3 disease (Nóbrega et al., 2015).

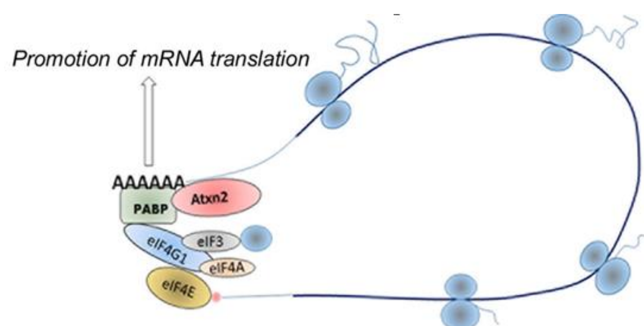

Figure 4: Promotion of mRNA translation by ataxin-2 protein. The interaction of ataxin-2 with polyribosomes (in the 3' untranslated regions) through the PABP protein leads to translation promotion, which suggests that this is one of the functions of ataxin-2 protein [Adapted from (Velázquez-Pérez et al., 2017)].

The ataxin-2 protein can also, under stress conditions, interact with compounds of the microRNA pathway, Me31B (Deadbox helicase Me31B) and Ago1 (Argonuate 1), and lead to translational suppression of mRNA, through the formation of stress granules (Figure 5) (Nonhoff et al., 2007).

The study carried out in *Caenorhabditis elegans* showed that, under adverse conditions, the ataxin-2 protein, associated with stress granules, inhibits the translation of some proteins, by blocking translation initiator factor, EiF4F (eukaryotic initiation factor

4F) (Tharun, 2009, Villacé et al., 2004, Ciosk et al., 2004, Kedersha and Anderson, 2002, Ralser et al., 2005b).

Another study demonstrated that the homolog of ataxin-2 protein in yeats is involved in the formation of stress granules (Kedersha and Anderson, 2002, Vernet and Artzt, 1997, Albrecht et al., 2004). Thus, appears that ataxin-2 protein has an important role in the negative regulation of translation.

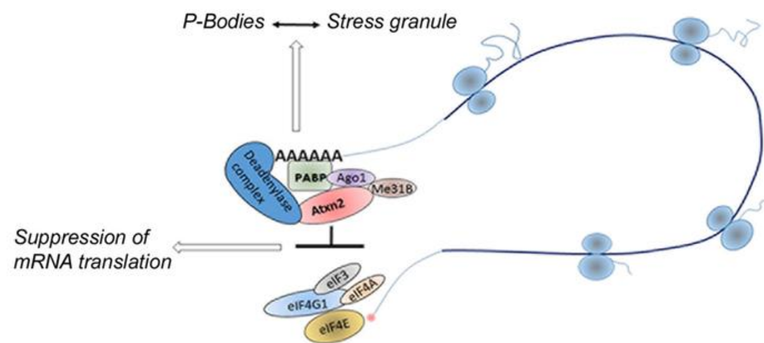

Figure 5: Suppression of mRNA translation by ataxin-2 protein. The ataxin-2 protein interacts with Me31B and Ago1 (microRNA pathway compounds) and blocks the translation of messenger mRNA through the formation of stress granules [Adapted from (Velázquez-Pérez et al., 2017)].

1.2.5.3. Metabolic modulation

Several studies have shown that ataxin-2 is involved in the modulation of metabolism, since an increase in this protein is related to weight loss and insulin sensitivity, while a decrease in ataxin-2 leads to insulin resistance, dyslipidemia, fat accumulation and obesity (Carmo-Silva et al., 2017). There are also, some SCA2 patients who report an increase in their appetite (Auburger, 2012, Abdel-Aleem and Zaki, 2008).

Ataxin-2 protein, under conditions of deficient nutrition, appears to be involved in the modulation of metabolism, by blocking the mammalian target of rapamycin (mTOR) pathway (metabolic pathway involved in obesity and insulin resistance) (Carmo-Silva et al., 2017). Ataxin-2 protein can block mTOR directly through mTOR mRNA sequestration into stress granules or indirectly through decrease in phosphorylation of RPS6K (ribosomal protein S6) and 4E-BP (eukaryotic initiation factor 4E-binding

protein), which are two important substrates in the mTOR pathway (Figure 6) (Lastres-Becker et al., 2016, DeMille et al., 2015, Albert and Hall, 2015).

Figure 6: Metabolic modulation by protein ataxin-2. Ataxin-2 protein can block the mTOR pathway, a metabolic pathway related to obesity and insulin resistance, either directly by sequestration of mTOR into stress granules or indirectly by promoting decreased phosphorylation of important substrates required in the mTOR pathway (RPS6 and 4E-BP) [Adapted from (Hofmann et al., 2021)].

Studies in *Caenorhabditis elegans* showed that a decrease in the expression of the homolog *ATXN2* gene causes fat accumulation, which is not reversed with caloric restriction (Carmo-Silva et al., 2017).

A recent study using ataxin-2 knockout mice, which are characterized by being obese and insulin resistant, demonstrated that overexpression of ataxin-2 prevents high-fat diet-induced obesity. Furthermore, it also demonstrated that normalizing ataxin-2 levels improves metabolic dysfunction without altering body weight and that clock genes may play an important role in this metabolic modulation (Carmo-Silva et al., 2022).

1.2.5.4. Calcium-mediated signalling

Calcium ions are responsible for the activation of ion channels and play an important role in cell homeostasis, especially in Purkinje cells, once they have a wide variety of calcium-dependent proteins and enzymes (Egorova and Bezprozvanny, 2019).

Ataxin-2 protein regulates the calcium-mediated signalling cascades by controlling the translation of certain components, such as ATP2a2 (ATPase Sarcoplasmic/Endoplasmic Reticulum Ca_2^+ Transporting 2), Inpp5a (Inositol Polyphosphate-5-Phosphatase A) and rora (RAR Related Orphan Receptor A) genes (Figure 7) (Pulst et al., 1996, Halbach et al., 2017). Thus, it appears that ataxin-2 protein has an important role in the calcium-mediated signalling and in the homeostasis of Purkinje cells.

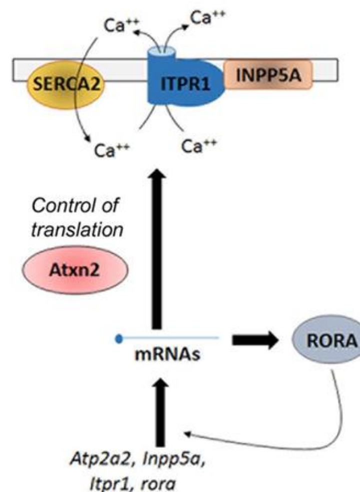

Figure 7: Calcium-mediated signalling by ataxin-2 protein. The ataxin-2 protein regulates the calcium signalling pathway by controlling the translation of some of the components that have a regulatory action in the Ca^{2+} release from the endoplasmic reticulum to the cytoplasm [Adapted from (Velázquez-Pérez et al., 2017)].

1.2.5.5. Endocytosis and cytoskeleton reorganization

Another function of the ataxin-2 protein seems to be its involvement in endocytosis and reorganization of the actin cytoskeleton. Studies in *Caenorhabditis elegans* and *Drosophila melanogaster* have showed that the ataxin-2 protein interacts with endophilins, which are responsible for inducing the curvature of the membrane in endocytosis sites and formation of actin filaments (Figure 8) (Ralser et al., 2005b, Satterfield et al., 2002, Gnazzo et al., 2016, Stubenvoll et al., 2016). In addition, ataxin-2 protein also interacts with parkin, which is responsible to modulate endocytic process, through the regulation of endosomal function and structure (Ralser et al., 2005b, Fallon et al., 2006, Huynh et al., 2003).

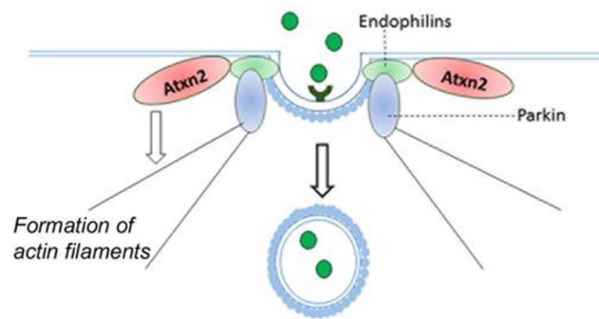

Figure 8: Endocytosis and cytoskeleton reorganization. The ataxin-2 protein interacts with endophilins and parkin, which are responsible for endocytosis processes and for reorganization of the cytoskeleton through the formation of actin filaments. [Adapted from (Velázquez-Pérez et al., 2017)].

1.2.6. Pathogenesis mechanisms of SCA2

The pathway responsible for neurodegeneration in polyQ diseases remains to be completely discovered and described. A better understanding of the molecular mechanisms involved in the pathogenesis of the disease is necessary to allow the development of better therapeutic strategies for the treatment of SCA2 disease (Egorova and Bezprozvanny, 2019). However, some discoveries have already been made about some of the mechanisms that appear to be involved in this disease and are explained in the sections below.

The CAG expansion in this disease leads to a mutant ataxin-2 protein with conformational changes, which results in a toxic gain of function. Thus, the mutant ataxin-2 protein triggers several pathogenic mechanisms (Figure 9), such as, protein oligomerization and aggregation, protein recycling, calcium homeostasis dysregulation, mitochondrial dysfunction and oxidative stress, dysregulation of transcription, proteolytic cleavage, posttranslational modifications, aberrant protein-protein interactions, axonal transport disruption and quality-control mechanisms dysfunction, which may contribute to the pathogenesis of the disease (Nóbrega and Almeida, 2012, Alves-Cruzeiro et al., 2016, Velázquez-Pérez et al., 2017).

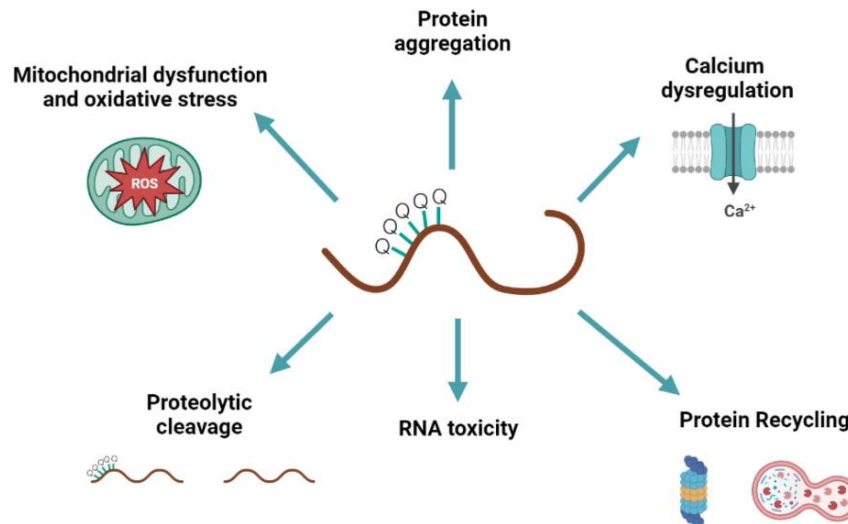

Figure 9: Pathogenesis mechanisms of SCA2. The pathogenic pathway responsible for neurodegeneration in polyQ diseases remains unclear, however appear to be several mechanisms involved, such as protein aggregation, calcium dysregulation, mitochondrial dysfunction and oxidative stress, proteolytic cleavage, RNA toxicity and protein recycling. [Adapted from (Gatchel and Zoghbi, 2005)]

1.2.6.1. Protein aggregation

Protein aggregation is a hallmark feature of SCA2 disease, however, the role of ataxin-2 aggregates and inclusions in the neurodegenerative process of SCA2 remains unclear. (Todd and Lim, 2013, Williams and Paulson, 2008, Takahashi et al., 2010, Bauer and Nukina, 2009). When an expansion of the number of CAG repeats occurs, the ataxin-2 protein presents an abnormal conformation, leading to the formation of aggregates, which can subsequently trigger different processes and lead to cell death and degeneration of structures in the central and peripheral nervous system (Matilla-Dueñas et al., 2010).

Exist two types of aggregates: granular cytoplasmatic staining (GCS) and neuronal nuclear inclusions (NNI). Some studies revealed that cytoplasmatic ataxin-2 granules are present in the first stages while nuclear inclusions are related to final stages of the disease. Furthermore, studies have revealed that GCS are related to severe neurodegeneration, while the presence of NNI appears to be associated with a protective function (Seidel et al., 2017, Ueda et al., 2014, Koyano et al., 2014).

1.2.6.2. Calcium dysregulation

In the cytoplasm, increased calcium results in apoptosis, and abnormal calcium-mediated signalling leads to dysregulation of cellular homeostasis, synaptic loss, and even cell death. It has already been shown that calcium-mediated signalling is altered in several neurodegenerative diseases, such as HD, SCAs and Alzheimer's disease (Chopra and Shakkottai, 2014, Egorova et al., 2015, Hisatsune et al., 2018, Egorova and Bezprozvanny, 2018).

Studies using SCA2-trangenic mouse demonstrated that the mutant ataxin-2 protein affects calcium-mediated signalling cascades, leading to an increase of glutamate and neuronal apoptosis, through its interaction with the C-terminal region of Itpr1 (inositol (1,4,5)-triphosphate type-1 receptor), which is a receptor located in the endoplasmic reticulum membrane (mainly in the Purkinje cells of the cerebellum) and regulates calcium release from the endoplasmic reticulum into the cytoplasm (Satterfield et al., 2002, Huynh et al., 2000, Zambonin et al., 2017) .

Thus, it appears that one of the mechanisms of SCA2 disease is related to an impairment of calcium signalling and an increase of glutamate, leading to the neurodegeneration associated with this type of disease (Huynh et al., 2000, Magaña et al., 2013).

1.2.6.3. Mitochondrial dysfunction and oxidative stress

There is some evidence that mitochondrial dysfunction plays a role in the pathogenesis of polyQ diseases (Panov et al., 2002).

In the case of SCA2, fibroblasts from patients revealed an increase expression of superoxide dismutase and a reduction in the expression of catalase (an enzyme that catalyses the decomposition of hydrogen peroxide). Thus, was observed an increase of intracellular hydrogen peroxide, which caused, among others, a dysfunction in mitochondrial activity and an increase oxidative stress (Cornelius et al., 2017).

1.2.6.4. RNA toxicity

In SCA2 disease, the mechanisms of RNA toxicity appear to be based on the production of bidirectional transcripts of the ataxin-2 gene, which lead to neurodegeneration. There is significant evidence that support this hypothesis, as expanded CAG repeats were detected in *post-mortem* cerebellum and cortex of SCA2 patients, in human fibroblasts and in human induced pluripotent stem cells (Li et al., 2016).

1.2.6.5. Proteolytic cleavage

In several polyQ diseases, it appears that the mechanism of pathogenesis is also related to proteolytic cleavage, which results in the formation of toxic fragments of polyQ proteins (Paulson et al., 1997, Merry et al., 1998).

Cleavage of the ataxin-2 protein can result in the production of a smaller fragment, which includes the N-terminal of the protein (containing the polyQ tract), and a larger fragment, which can alter cellular functions, such as splicing (due to the C-terminal tendency to bind to the A2BP1 protein) (Turnbull et al., 2004). Studies demonstrate that in human cortical brain, ataxin-2 is found in two fragments and in SCA2 patients these fragments have a higher expression, thus suggesting a possible link between ataxin-2 fragmentation and SCA2 pathogenesis (Matos et al., 2017).

1.2.6.6. Protein recycling

The mutant protein, with an expended CAG tract, is toxic and associated with neuronal cell death, therefore it must be eliminated (Jimenez-Sanchez et al., 2012). The clearance of these proteins can occur in two ways, the ubiquitin-proteosome system (UPS) or autophagy (Rubinsztein, 2006). However, in several neurodegenerative diseases, the impairment of protein recycling (UPS and autophagy), results in neurotoxicity and neurodegeneration (Jimenez-Sanchez et al., 2012, Cortes and La Spada, 2015, Matsuda and Tanaka, 2010, Takalo et al., 2013).

Studies have shown that in SCAs, both UPS and autophagy are compromised. Thus, it is important to understand how this protein recycling process works, in order to

be able to use it as a therapeutic target in SCA2 disease (Damrath et al., 2012, Halbach et al., 2015, Paul et al., 2018).

1.2.6.6.1. Ubiquitin-proteosome system

The ubiquitin-proteosome system (UPS) is a degradative pathway, which targets substrates for proteasomal degradation by linking them to a polyubiquitin chain (Woelk et al., 2007).

Ubiquitination, in addition to marking substrates for degradation, is also responsible for endocytosis, DNA (deoxyribonucleic acid) repair, protein trafficking and cell division. The ubiquitination process consists in three sequential reactions catalyzed by three different enzymes, starting with ubiquitin activation by ubiquitin-activating enzyme, then moving on to ubiquitin-conjugating enzyme and finally to ubiquitin-protein ligase, which binds the activated ubiquitin to a specific target (Pickart, 2001, Hamilton and Zito, 2013, Woelk et al., 2007, Nandi et al., 2006). This ubiquitination process is repeated several times leading to the formation of a polyubiquitin chain and, depending on the size of this ubiquitin chain, this substrate can be directed to different locations of the cell, for example, a chain with 48 ubiquitin's is directed to proteasomal degradation, while a chain of 63 ubiquitin's is targeted towards lysosomal degradation (Hamilton and Zito, 2013, Woelk et al., 2007).

Studies have shown that in polyQ disease, proteins with a polyglutamine tract are not degraded efficiently by UPS, resulting in fragments, that appear to be even more toxic (described in detail in the section above: 1.2.6.5 – Proteolytic cleavage) (Holmberg et al., 2004, Venkatraman et al., 2004).

1.2.6.6.2. Autophagy

Autophagy is a catabolic process, which functions normally works at a low level, however, in certain situation, such as nutrient deficit, DNA or organelle damaged and accumulation of abnormal proteins is highly induced. Thus, in polyQ diseases like SCA2, in which accumulation of misfolded proteins occurs, autophagy may play an important role in the clearance of mutant proteins (Fujikake et al., 2018).

Autophagy can be divided into three distinctive mechanisms, macroautophagy, microautophagy and chaperone-mediated autophagy (Figure 10) (Fujikake et al., 2018).

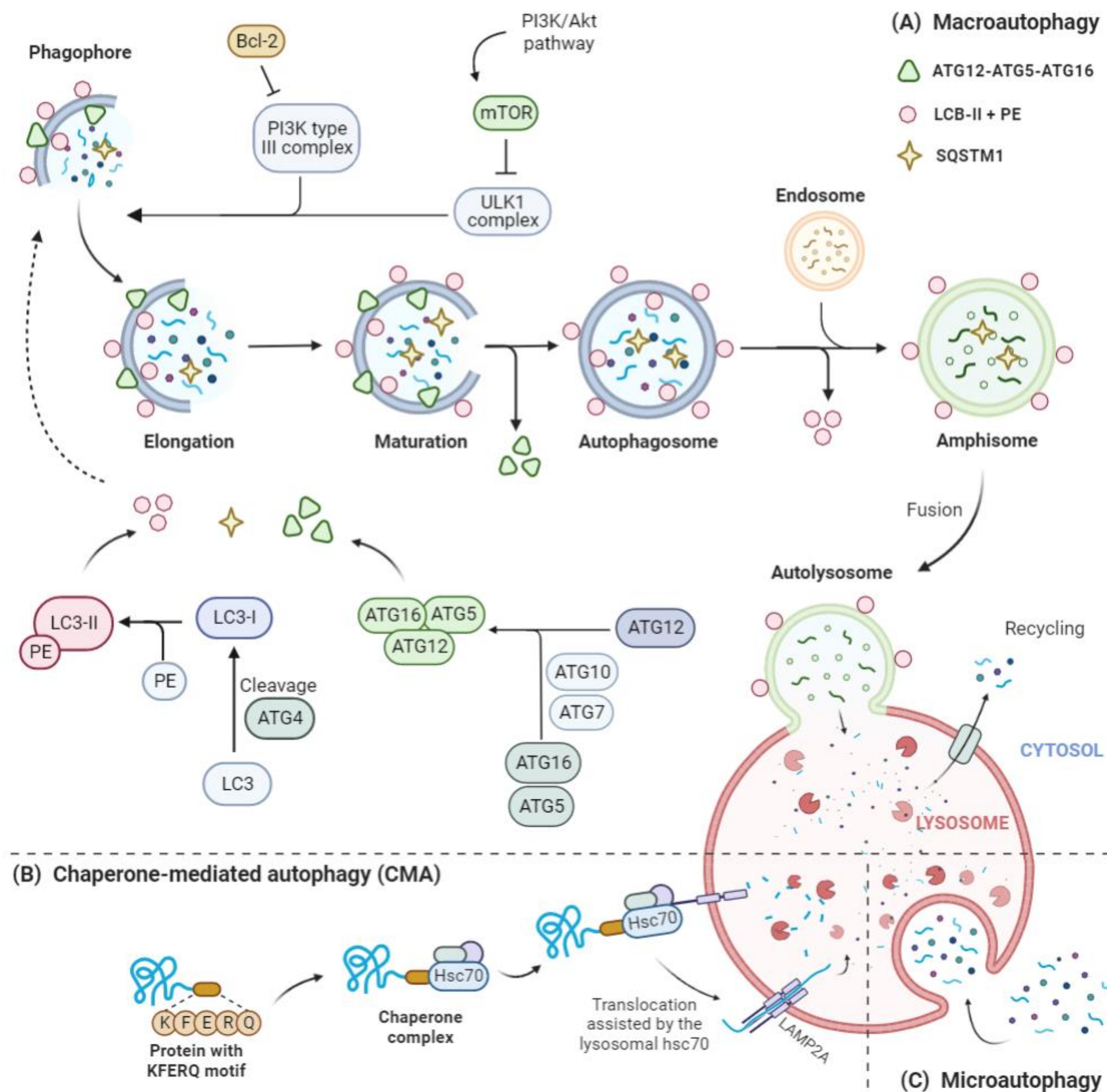

Figure 10: Different mechanisms of autophagy (macroautophagy, chaperone-mediated autophagy and microautophagy). The macroautophagy pathway consists in three phases, phagophore formation, autophagosome elongation and formation, and autophagosome maturation and fusion with lysosomes, forming the autolysosome, where degradation occurs. Several proteins are involved in this process: SQSTM1 directs ubiquitinated substrates to the autophagosome, PI3K type III complex is responsible for phagophore nucleation, PI3K type I suppresses autophagy through the inactivation of Ulk1 by mTOR, the Atg12–Atg5·Atg16L complex is essential for autophagosome formation and LC3B-II is responsible for autophagosome maturation **(A)**. Chaperone-mediated autophagy is based on the recognition of substrates with KEFRQ

motif by Hsc 70. After recognition, the substrate is directed to the lysosome, interacts with the LAMP2A receptor, and enters into the lysosome, where it is degraded **(B)**. In microautophagy, the cellular constituents are degraded directly by engulfment of the lysosomal membrane **(C)**. [Adapted from (Sarkar, 2013)].

1.2.6.6.2.1. Macroautophagy

Macroautophagy (usually named only by autophagy) consists in engulfment of cytoplasmic membrane into a double-membrane vesicle called autophagosome, which then fuses with the lysosome and protein degradation occurs. Several proteins are involved in this process, among them are sequestosome protein 1 (SQSTM1; recognizes ubiquitinated substrates and directs them to autophagosomes) and microtubule-associated protein 1 light chain 3 Beta (LC3B; responsible for autophagosome maturation) (Figure 10A) (Jimenez-Sanchez et al., 2012, Onofre et al., 2016, Ravikumar et al., 2010, Fujikake et al., 2018, Lee and Lee, 2016, Katsuragi et al., 2015).

The macroautophagy pathway consists of several phases, starts with the formation of the phagophore, following by the elongation and fusion forming a double-membrane vesicle, the autophagosome, and finally occurs maturation and fusion of the autophagosome with lysosomes, forming the autolysosomes, where the degradation of its content by hydrolases occurs (Gordon and Seglen, 1988, Kuma et al., 2004).

In the first phase of macroautophagy, the phagophore is formed by lipid contribution of cellular components, such as endoplasmic reticulum, golgi, mitochondria, and the plasma membrane through clathrin-mediated endocytosis (Axe et al., 2008, Ravikumar et al., 2010, van der Vaart et al., 2010, Geng et al., 2010, Hailey et al., 2010). In this phase, autophagy is regulated by PI3K (phosphatidylinositol 3-kinase) type I and III. On the one hand, PI3K type I indirectly activates mTOR, which in turn suppresses autophagy, through inactivation of Ulk1 (Unc-51-like kinase) (Figure 10A). On the other hand, a multiprotein complex, that includes PI3K type III and beclin-1 is responsible for phagophore nucleation and can be inhibited by Bcl2 (B-cell lymphoma 2 protein) and thus block the autophagy (Axe et al., 2008, Fujikake et al., 2018).

After the formation of the phagophore, the membrane will grow (elongation) and subsequently fuses to form a double membrane vesicle, named autophagosome. One of the proteins involved in this phase are Atg9 (autophagy-related protein 9; a

transmembrane protein implicated in membrane delivery) and two ubiquitin-like reactions are required (Takahashi et al., 2011, Young et al., 2006). In the first reaction, there is the formation of Atg12–Atg5·Atg16L complex, trough Atg7 and Atg10 actions. The Atg12–Atg5·Atg16L complex is located on the outside of the membrane but dissociates when the membrane fuses and form the autophagosome (Figure 10A) (Mizushima et al., 1999, Mizushima et al., 1998, Mizushima et al., 2002, Mizushima et al., 2003, Tanida et al., 2001).

In the other reaction, the formation LC3B-II occurs, by cleavage of LC3B-I through Atg4 and then by its conjugation with PE (phosphatidylethanolamine) (Figure 10A) (Tanida et al., 2002, Hemelaar et al., 2003). LC3B-II is involved in autophagosome maturation and serves as a protein adapter in selective autophagy (Lee and Lee, 2016). This is located on both sides of the membrane and, even after fusion with lysosomes, remains associated with the membrane, so it can be used as an autophagy measure because we can correlate the number of autophagosomes in the cell with the levels of LC3B-II (Rubinsztein et al., 2009, Klionsky et al., 2008, Kabeya et al., 2000).

Finally, in the maturation phase, autophagosomes can fuse with endosomes and/or lysosomes and form amphisomes and/or autolysosomes respectively. Autophagosomes move along microtubules and towards lysosomes, however, under starvation conditions, pH increases, causing the lysosome to move and get in the way of the autophagosome, facilitating the fusion between the two. After the fusion, within autophagolysosome the degradation process by hydrolases occurs (Berg et al., 1998, Gordon and Seglen, 1988, Jahreiss et al., 2008, Kimura et al., 2008, Korolchuk et al., 2011).

In addition, autophagy can be regulated through dependent or independent mTOR pathway (Jimenez-Sanchez et al., 2012).

Regulation via mTOR-dependent pathway: On the one hand, in nutrient deprivation, the AMPK (adenosine monophosphate-activated kinase) becomes active leading to inactivation of the mTOR pathway and therefore the activation of autophagy (since mTOR stops inhibiting ULK1). Furthermore, under these conditions, AMPK can also directly activate the ULK1 complex and induce the autophagy process. On the other hand, under nutrient-rich conditions, mTOR suppresses autophagy through a

phosphorylation that inhibits the ULK1 complex (Inoki et al., 2003, Hosokawa et al., 2009, Kim et al., 2011, Egan et al., 2011).

There are some autophagy inducers, that act by inhibiting mTOR, such as rapamycin, which are being studied in an attempt to use this pathway as a therapeutic target for polyQ diseases (Ravikumar et al., 2002, Ravikumar et al., 2004). Studies in SCA3 mouse models, in which a rapamycin analog was used to increase autophagic activity, showed a reduction of aggregates in the brain and an improvement in motor dysfunction (Menzies et al., 2010).

Regulation via mTOR-independent pathway: The increase in cAMP (cyclic adenosine monophosphate) by adenylate cyclase leads to an increase in Ins(1,4,5)P_3 [(IP_3); through activation of Epac (exchange factor directly activated by cAMP)], which indirectly stimulates the hydrolysis of PIP_2 (phosphatidylinositol 4,5-bisphosphate) into IP_3. This can directly inhibit autophagy or interact with endoplasmic reticulum membrane receptors, leading to calcium release. The increase of intracellular calcium leads to inhibition of autophagosome maturation and activation of calpains, which block autophagy (Williams et al., 2008, Sarkar et al., 2005).

There are some autophagic modulators, such as lithium, sodium valproate and carbamazepine, which induce the autophagic process by decreasing IP3 levels. Studies in HD cell models showed decreased aggregation of mutant huntingtin after the use of these modulators (Williams et al., 2008, Sarkar et al., 2005, Williams et al., 2002, Sarkar et al., 2008).

1.2.6.6.2.2. Chaperone-mediated autophagy

Chaperone-mediated autophagy is a specific mechanism, since only cytoplasmic substrates that are coupled to a targeting KFERQ motif (lysine-phenylalanine-glutamic acid-arginine-glutamine like pentapeptide motif) are degraded. This type of autophagy consists in the recognition of soluble proteins, that contain the KEFRQ motif, by heat shock cognate protein of 70 kDa (Hsc70). After recognition, the substrate is directed to the lysosome membrane and interacts with the membrane receptor, LAMP2A (lysosome-associated membrane protein type 2A). After this interaction, the substrate is unfolded and enters in the lysosome, where degradation will occur (Figure 10B) (Kaushik et al., 2011, Kaushik and Cuervo, 2012)

1.2.6.6.2.3. Microautophagy

Microautophagy is characterized by invaginations of the lysosomal membrane to engulf cellular constituents, which are subsequently degraded in the lysosomal lumen (Figure 10C) (Li et al., 2012).

1.2.7. Therapeutic opportunities for SCA2

There is still no effective therapy to stop or delay the disease progression for SCA2 patients. Currently, the only treatments available are symptomatic and supportive. So, after diagnosis, SCA2 patients should be followed up through periodic medical visits with the aim of evaluating disease progression and appearance of new symptoms, which can be alleviated through pharmacologic or non-pharmacologic treatments (Table II). In addition, monitoring the progression of the disease helps to predict how the disease may progress or even the possibility of the patient being able to participate in a clinical trial. Over time is also important discuss palliative care with the patient and their family (Klockgether et al., 2019).

Advances and discoveries have been made regarding new therapeutic options, however there are some factors that make the discovery of an effective therapy difficult, for example, SCA2 is a rare disease and therefore not attractive enough for most pharmaceutical companies, clinical trials are carried out with few people and in an advanced stage of the disease (extended neuronal degeneration) and, finally, due to the lack of a good biomarker (predictive and progression) to assess the effectiveness of the therapy (Velázquez-Pérez et al., 2017)

Table II

Pharmacological or non-pharmacological therapy available for SCA2 disease

Pharmacological treatment	Symptom	Non-pharmacological treatment
Selective serotonin reuptake inhibitors, mood-stabilizing antiepileptic drugs, benzodiazepines, and neuroleptic drugs	**Behavioural abnormalities**	Not available
Tetrabenazine and neuroleptic drugs	**Chorea**	Not available
Selective serotonin reuptake inhibitors	**Depression**	Psychological support
Not available	**Dysphagia**	Logopaedic rehabilitation, dietary modification, and percutaneous endoscopic gastrostomy
Benzodiazepines, trihexyphenidyl biperiden and focal intramuscular botulinum toxin	**Dystonia**	Deep brain stimulation
Pregabalin. Gabapentin, carbamazepine, and duloxetine	**Neuropathic pain and paraesthesia**	Acupuncture
Levodopa and dopamine agonists	**Parkinsonism**	Not available
Not available	**Respiratory sleep apnoea**	Non-invasive ventilation
Benzodiazepines and pramipexole	**Restless leg syndrome**	Not available
Antiepileptic drugs and benzodiazepines	**Seizures and myoclonus**	Not available
Benzodiazepines, zolpidem, melatonin, trazadone, and mirtazapine	**Sleep disturbances**	Not available
Baclofen, eperisone, tizanidine, benzodiazepines and focal intramuscular botulinum toxin	**Spasticity**	Physiotherapy and stretching
Anticholinergic drugs, α1-selective α-blockers, and β_3-adrenergic receptor agonists	**Urinary disturbances**	Urinary catheterization

[Adapted from (Klockgether et al., 2019)]

1.2.7.1. Physical rehabilitation

Despite the possibility of using other therapeutic options, physical rehabilitation should always be performed in patients with neurodegenerative diseases. In Cuba, from patients with SCA2 undergoing this type of therapy, it is estimated that 68% improved their quality of life through the improvement of some clinical parameters (Magaña et al., 2013, Pérez-Avila et al., 2004, Velázquez-Pérez et al., 2011b).

1.2.7.2. Pharmacological therapy

Studies have shown that dopaminergic and anticholinergic treatments have improved symptoms such as tremor, dystonia and bradykinesia in SCA2 patients, while treatments with magnesium, quinine, mexiletine or vitamin B (high doses) help with muscle cramps (Pirker et al., 2003, Freund et al., 2007). Beyond this, treatments with levodopa alleviate rigidity and bradykinesia and treatments with coenzyme Q10 demonstrate improvements in oxidative stress of fibroblasts from SCA2 patients (Lastres-Becker et al., 2008, Furtado et al., 2004, Cornelius et al., 2017).

In 2006, in a clinical trial (double blind) carried out with 36 Cuban SCA2 patients and administering 50 mg of zinc sulfate or placebo for 6 months, it was verified that the group that received the treatment had slight improvements in cerebellar syndrome, peripheral neuropathy, saccade pathology, and oxidative stress, when compared with the group of patients who received placebo treatment (Velázquez-Pérez et al., 2011a).

Another study with 20 SCA2 Cuban patients administering high doses of vitamin B was carried out and a significant decrease in muscle cramps, cognitive partial recovery and improvements of clinical and electrophysiological markers of peripheral neuropathy were demonstrated (Pérez et al., 2014).

A recent study (double blind/placebo) carried out with 34 SCA2 patients and administering NeuroEPO (human-recombinant erythropoietin) nasally during 6 months demonstrated the safety and tolerability of NeuroEPO and suggested, in treated patients, effects in motor and cognitive abnormalities, however confirmatory studies are necessary (Rodriguez-Labrada et al., 2022).

1.2.7.3. Calcium stabilizers

One of the mechanisms of SCA2 disease is the increased release of calcium from the endoplasmic reticulum to the cytoplasm. Therefore, this therapy is based on the modulation of proteins that regulate calcium homeostasis, with the aim of normalizing calcium levels, leading to an improvement of Purkinje cells functions (Egorova and Bezprozvanny, 2019).

Several studies were performed in SCA2 mice and using calcium stabilizers (such as rianudin, dantrolene, riluzole, 5PP, NS13001 and CHZ), which normalized calcium, leading to decreased loss of Purkinje cells and improved motor dysfunction in these mice (Liu et al., 2009, Kasumu et al., 2012b, Bushart et al., 2018, Kasumu et al., 2012a, Cho et al., 2018).

1.2.7.4. Gene silencing

Another strategy to decrease mutant ataxin-2 protein levels is target gene silencing. This silencing can be performed through RNA interference (RNAi) or antisense oligonucleotides (ASOs), but there is an obstacle, because in humans, silencing can lead to knockdown of both alleles (normal and mutant). Nevertheless, through single nucleotide polymorphisms it is possible to make a specific silencing for the mutant transcript, while the normal remains intact (Magaña et al., 2013).

1.2.7.4.1. RNA interference

RNAi is based on a cellular mechanism of gene expression silencing, which reduces the levels of the target protein, through small sequences of non-coding RNA, such as siRNAs (synthetic small interfering RNA), shRNAs (short hairpin RNA) and microRNAs. This gene silencing method has been used in several studies involving polyQ diseases, such as SCA1, SCA3, SCA7 and HD (Sullivan et al., 2019, Kim and Rossi, 2008, Xia et al., 2004, Rodriguez-Lebron et al., 2005, Franich et al., 2008, Machida et al., 2006, Wang et al., 2005, Caplen et al., 2002, Koutsilieri et al., 2007, Nóbrega et al., 2013, Nóbrega et al., 2014, Conceição et al., 2016, Nóbrega et al., 2019).

A study in SCA1 transgenic mouse and using shRNAs for ataxin-1 demonstrated improvements in motor coordination, decreased accumulation of mutant ataxin-1 and restoration of cerebellar morphology (Xia et al., 2004).

In mouse models of SCA3 disease and after disease onset, administration of viral vectors encoding shRNAs, which silence mutant ataxin-3, led to improvement in motor behavior deficits and neuropathological symptoms when compared to controls (Nóbrega et al., 2013). Furthermore, a similar study in which silencing was performed at a pre-symptomatic stage demonstrated a decrease in the formation of ataxin-3 mutant aggregates and the associated neuronal dysfunction. In this study, it was also observed that silencing prevented the progression of balance and motor coordination deficits in treated animals when compared to controls (Nóbrega et al., 2014).

1.2.7.4.2. Antisense oligonucleotide therapy

Antisense nucleotides (ASOs) are one of the most promising therapies for the treatment of neurodegenerative diseases such as SCA2 disease. ASOs are single-stranded DNA sequences and act through their complementarity binding to RNA transcripts (gene-targeting effect). The DNA-RNA complex is cleaved by ribonuclease H, leading to non-translation of mRNA and reduced levels of toxic protein (mutant ataxin-2) (Figure 11) (Gheibi-Hayat and Jamialahmadi, 2021, Schoch and Miller, 2017, Geary et al., 2015).

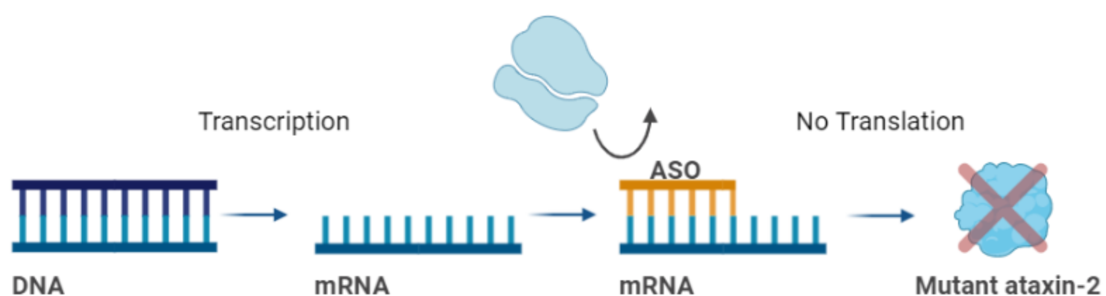

Figure 11: Antisense oligonucleotide therapy. After transcription, ASO binds by complementarity to the target mRNA (ataxin-2) and leads to non-translation of mRNA and subsequently to reduced levels of mutant ataxin-2 [Adapted from (Egorova and Bezprozvanny, 2019)].

Studies revealed that ASO therapy reduced mutant ataxin-2 levels and led to a decrease in the progression of motor symptoms and an increase in the survival of transgenic mice (Becker et al., 2017). ASO therapy has also shown promising results in clinical trials involving HD patients, so this therapy could be a possible future therapy for patients with polyQ diseases, including SCA2 disease (van Roon-Mom et al., 2018).

1.2.7.5. Stem cells therapy

Stem cells therapy is based on extracting cells from a healthy donor and administering them to SCA2 patients to regenerate the affected neurons (Figure 12). Stem cell-based therapies have already been performed in several SCA mouse models (Egorova and Bezprozvanny, 2019).

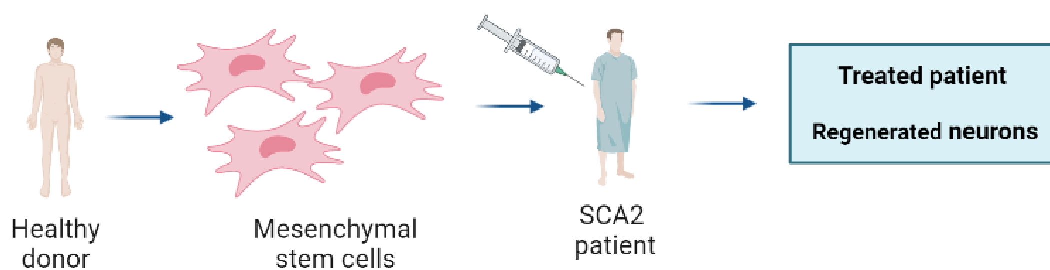

Figure 12: Stem cells therapy. This therapy is based on the extraction of hMSCs from a healthy donor and administration to a SCA2 patient. These cells release neurotrophic factors and have an immunomodulatory function leading to the regeneration of affected neurons. [Adapted from (Egorova and Bezprozvanny, 2019)].

In studies with SCA2 models, where human mesenchymal stem cells (hMSCs) were injected, there was a decrease in disease progression (motor function) due to decreased loss of Purkinje cells in the cerebellum. hMSCs are capable of differentiate into different cell types and release some neurotrophic factors, which can improve Purkinje cells survival (Chang et al., 2011)

In Taiwan, a clinical trial was conducted with this therapy. hMSCs from healthy donors were intravenously injected into six patients with SCA3. After nine months, these patients showed some improvements, however, further studies are needed to prove the possibility of using this therapy in this type of disease (Tsai et al., 2017).

1.2.7.6. Decreased ataxin-2 protein aggregates

One of the mechanisms of SCA2 disease pathogenesis is related with the formation of ataxin-2 aggregates due to the abnormal expansion of glutamines in the causative proteins. Thus, a possible future therapeutic option may be related to the reduction of these aggregates, by reducing their formation or the increasing their destruction (Magaña et al., 2013).

On the one hand, the decrease in protein aggregates can be achieved through the inhibition of aggregation by molecules that destabilize the structure of the aggregates, molecules that function competitively (blocking the binding of aggregates to ataxin-2 monomers), or through the increase of chaperones, such as heat shock protein 70 (Tanaka et al., 2008, Sánchez et al., 2003, Dedeoglu et al., 2002, Igarashi et al., 1998, Friedman et al., 2008, Wyttenbach, 2004). In SCA1 mice, the overexpression of Hsp70 (heat shock protein 70) led to motor improvement and suppression of the neuropathological phenotype (Cummings et al., 2001).

On the other hand, the number of aggregates may decrease due to increased aggregate destruction, which can be accomplished through UPS or autophagy stimulation (Matilla-Dueñas et al., 2010). Studies in HD and SCA3 models showed that the use of compounds that stimulate the UPS system (benzamyl and Y-27632) caused a reduction in the aggregation and toxicity of proteins with CAG repeats expansion (Wong et al., 2008, Pollitt et al., 2003).

1.2.7.6.1. Cordycepin

Cordycepin or 3’-deoxyadenosine is a bioactive compound found in *Cordyceps militaris* (parasitic fungus widely used in traditional Chinese medicine), which in cells can be converted into mono-, di- or triphosphates (Klenow, 1963, Tuli et al., 2013). After entering the cell by adenosine transporters (ENT1/ENT2), adenosine kinase transforms cordycepin in cordycepin 5'-monophosphate (CoMP), which functions as an AMP (Adenosine monophosphate) analogue and therefore mimics its function in the AMPK pathway leading to AMPK activation and induction of autophagy (Figure 13) (Hawley et al., 2020)

Studies indicate that cordycepin can activate AMPK, which is responsible for several functions such as inactivation of the mTOR pathway, regulation of cell growth and proliferation, metabolic regulation (lipids and glucose), control of body weight and food intake, activation of autophagy (Figure 13), among others (Marcelo et al., 2019, Rosso et al., 2016).

Recently was shown that cordycepin through pharmacology activation of autophagy by AMPK activation (independently of mTOR) significantly reduce the levels of mutant ataxin-3, motor and neuropathological deficits in mouse model of SCA3 disease, and the number of ubiquitin aggregates in a human neural model (Marcelo et al., 2019).

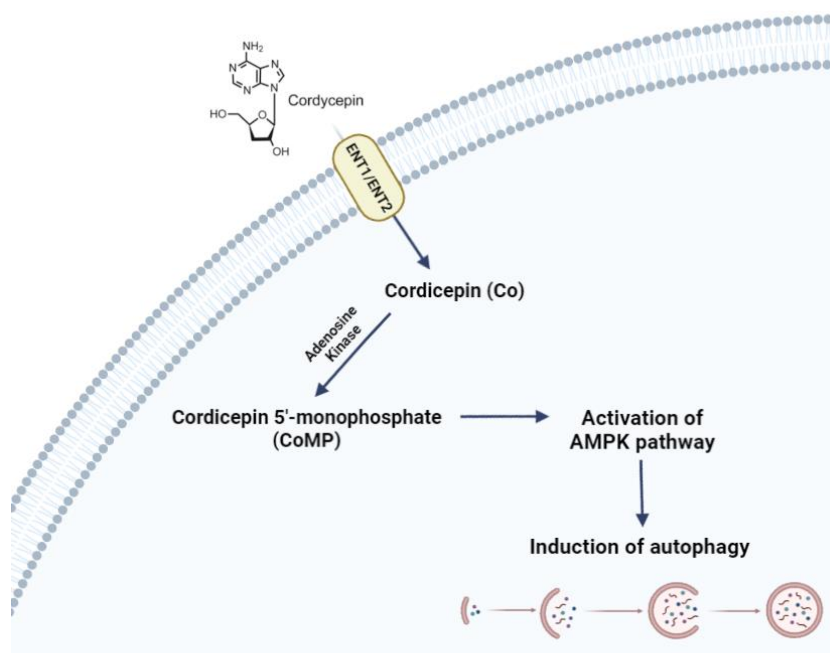

Figure 13: Mechanism of activation of AMPK by cordycepin. After entering the cell by ENT1/ENT2, adenosine kinase transforms cordycepin in CoMP, which is an AMP analogue and therefore leads to AMPK activation and induction of autophagy [Adapted from (Hawley et al., 2020)]

In polyQ diseases, such as SCA2, autophagy is important in the clearance of misfolded proteins and in the regulation of protein homeostasis, thus, cordycepin by the increase of autophagy may become an effective pharmacology therapy in the treatment of polyQ diseases (Marcelo et al., 2019, Matos et al., 2017).

2. Objective

SCA2 is an autosomal hereditary ataxia belonging to the polyQ diseases group and it is caused by an abnormal expansion of a CAG tract in the coding region of *ATXN2* gene, which translates into an abnormal glutamine stretch in the ataxin-2 protein, that cannot be efficiently degraded. One important neuropathological hallmark of SCA2 and of the other polyglutamine diseases is the presence of intraneuronal aggregates of the mutant protein in the brain (Matos et al., 2017).

Autophagy is an important cellular pathway of protein clearance, especially for aggregated misfolded proteins, which was shown to be deregulated in different neurodegenerative diseases. Moreover, it was proved to be a molecular target for therapeutic intervention in these diseases. Except for SCA2, in all the other polyQ diseases the autophagy pathway was shown to be impaired or dysfunctional (Cortes and La Spada, 2015).

Therefore, in this project, we aim to study the autophagy pathway in SCA2, by investigating several autophagy markers in cellular and animal models for the disease, and in postmortem brain tissue from SCA2 patients. Additionally, we aim to investigate if the upregulation of autophagy could be used as a therapeutic strategy for this disease.

3. Materials and Methods

In this project were used mouse neuroblastoma line-derived cells (Neuro-2A), mice brains, which were sliced with 20 µm of thickness, using a Cryostat (Cryostar NX50, ThermoFisher Scientific) and stored in freefloating PBS (phosphate buffer saline) with 0.05 µm sodium azide solution at 4ºC, and human post-mortem brain tissue from two clinically and genetically confirmed SCA2 patients (NIH NeuroBioBank, USA), stored in floating PBS/sodium azide solution at 4°C.

Statistical analysis was performed using Student's t-test or one-way ANOVA followed by post hoc Turkey's multiple comparison test using GraphPad software (La Jolla, USA). Significant thresholds were set at $P < 0.05$, $P < 0.01$ and $P < 0.001$.

3.1. Neuroblastoma Cell Culture

Neuro-2A were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco). When Neuro-2A cells reached about 75/80% confluency, were detached with trypsinization, divided, and incubated at 37 °C in an atmosphere with 5% carbon dioxide and saturated humidity.

3.1.1. Cell transfection

Twenty-four hours after cultured the cells in 12-well plates, transfection is performed by adding 3 µl PEI solution (polyethylenimine 1mg/ml, Tebu-bio) with 500ng of DNA plasmids in 10 µl DMEM without supplementation. Then the cells were incubated for 48 hours and collected for western blot (WB) assays. The DNA-constructs used were the following: human full-length ataxin-2 with an EGFP (enhanced green fluorescent protein) tag carrying normal polyQ segment (22Q: Atx2-Q22) or mutant polyQ segment (58Q: Atx2-Q58 or 104Q: Atx2-Q104).

For chloroquine (ChQ) experiments, 100 µM of 1mg/ml solution of ChQ (Sigma) in DMSO (dimethyl sulfoxide) was used and collected six hours after being treated with this solution.

3.1.2. Protein extraction and western blot

Neuro-2A cells extracts were lysed in 10x RIPA-buffer (radioimmunoprecipitation assay buffer) supplemented with protease inhibitors (Roche Pharmaceuticals), followed by ultrasound sonication. Protein concentration levels was determined with Pierce^{TM} BCA Protein Assay Kit (Thermo Scientific).

Protein extracts were resolved in sodium dodecyl sulphate polyacrylamide gels (7.5% stacking and 12% running). Proteins were transferred onto polyvinylidene difluoride membranes (Merck Millipore) according to standard protocols. Then, the membranes were blocked by incubation in 5% BSA (bovine serum albumin) in Tris-buffered saline containing 0.1% Tween 20 for one hour at room temperature, followed by overnight incubation at 4ºC with the primary antibodies diluted in blocking buffer. The antibodies used were the following: rabbit anti-LC3B (1:1000, ref. NB100-2220, Novus) and rabbit anti-SQSTM1 (1:1000, ref. 5114S, Cell Signalling). After incubation with the primary antibody, membranes were incubated with the secondary antibodies for two hours at room temperature, bands were visualized with Enhanced Chemiluminescent substrate (GE Healthcare) and ChemiDoc equipment (Bio-Rad). Semi-quantitative analysis was carried out based on the optical density of scanned membranes and the specific optical density was normalized with respect to the amount of beta-actin loaded in the corresponding lane of the same gel. Optical densiometric analysis was carried out using Image J software.

3.2. Cordycepin treatment *in vivo*

One week after mice were stereotaxically injected with lentivirus (encoding for ataxin-2 mutant) into the right hemisphere, treatment with cordycepin was initiated. Mice were randomly distributed in two groups: the treated mice were administrated with an intraperitoneal (IP) injection of 20mg/Kg of cordycepin in DMSO-NaCl 0.1% solution and the non-treated mice were administrated with an IP injection of the saline vehicle [Sodium chloride (NaCl) 0.1%]. The injections were performed according to the following schedule: twice a week for five weeks, three times a week for four weeks and finally, five times a week for three weeks. After the injections were completed, the mice were sacrificed for immunohistochemistry analysis.

The mice were weighed regularly to determine the concentration that would need to be administered of cordycepin and saline vehicle (Appendix I and II)

3.3. Immunohistochemistry (mouse and human *post-mortem* brain samples)

Immunohistochemistry assay began by incubating free floating the brain slices in phosphate buffer solution containing 0.1% phenylhydrazine for thirty minutes at 37°C followed by thirty minutes hydration in 0.1 PBS. Sections were then incubated in blocking solution [0.1% TritonX 100 containing 10% Normal Goat Serum (Gibco) in PBS] for one hour at room temperature, followed by overnight incubation at 4°C with respective primary antibodies. Posteriorly, sections were incubated with secondary antibody for two hours at room temperature. The secondary antibody (1:200, Vector antibodies) used was biotinylated and followed a reaction with the Vectastain elite avidin-biotin-peroxidase kit and by 3,3-diaminobenzidine substrate (both from Vector Laboratories). Sections were then mounted over microscope slides, hydrated with distilled water, and then dehydrated by passing through an increased degree of ethanol solutions (75, 96 and 100%) and finally xylene. Slides were covered with mounting medium Eukitt (O. Kindler GmbH & CO, Freiburg, Germany) and cover slipped. Images were acquired with 20x objective in a Zeiss Axio Imager Z2 and Axio Scan.Z1 Slide Scanner microscopes.

Samples were incubated with the following antibodies: mouse anti-ataxin-2 (1:1000, ref. 611378, BD Biosciences); rabbit anti-DARPP-32 (1:1000, ref. AB10518, Merck Millipore); rabbit anti-LC3B (1:1000, ref. NB100-2220, Novus); rabbit anti-SQSTM1 (1:1000, ref. 5114S, Cell Signalling).

4. Results and discussion

4.1. Dysregulation of autophagy in cells transfected with mutant ataxin-2

SCA2, like other polyQ diseases, is caused by an abnormal expansion of CAG repeats, which results in the formation of a protein, which tends to form aggregates. Thus, autophagy is an important cellular mechanism in this type of disease since it is responsible for the clearance of misfolded aggregated proteins. However, several studies have shown that autophagy is also compromised in various polyQ diseases (Alves-Cruzeiro et al., 2016, Damrath et al., 2012, Velázquez-Pérez et al., 2017, Halbach et al., 2015, Paul et al., 2018).

To understand whether autophagy is also compromised in SCA2 disease, the levels of autophagy markers, LC3B and SQSTM1, were analyzed to see how the autophagy pathway is affected in this disease.

Neuro-2a cells were transfected with different constructs of human ataxin-2, carrying different number of polyQ repeats: Atx2Q22 (WT form), Atx2Q58 and Atx2Q104 (MUT forms). After transfection, the protein levels of the autophagic markers, LC3B and SQSTM1, were analyzed by WB (Figure 14A). Through WB analysis we can verify that when cells express mutant ataxin-2 (Atx2Q58 and Atx2Q104) there is an increase of SQSTM1 levels when compared to the control condition (Figure 14B). If we compare only the values obtained for SQSTM1 between cells transfected with Atx2Q104 and cells transfected with Atx2Q22, we can verify that the increase in SQSTM1 in cells expressing Atx2Q104 is significant ($P<0.05$).

In contrast to SQSTM1, cells expressing Atx2Q58 and Atx2Q104 have a significant decreased levels of LC3B-II compared to non-transfected cells and cells transfected with wild-type ataxin-2 (Figure 14C).

We can also verify that non-transfected cells and cells transfected with wild-type ataxin-2 present similar levels of autophagic markers (LC3B and SQSTM1), proving that the difference of these cells when compared to the levels in cells that express Atx2Q58 and Atx2Q104 is not due to the transfection process, but only because the cells express mutant ataxin-2.

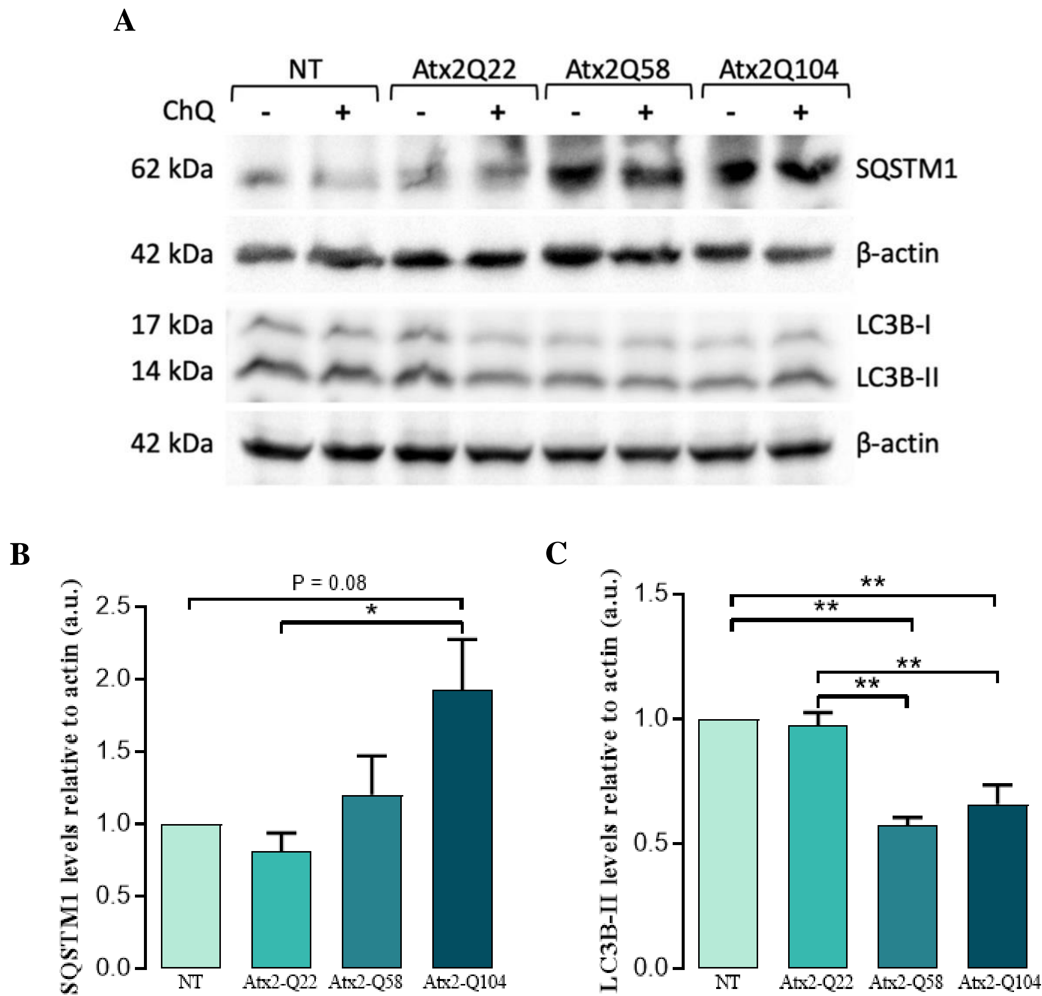

Figure 14: Alteration of autophagic markers in cells transfected with mutant ataxin-2 forms. Neuro-2A cells were transfected with human wild-type ataxin-2 (Atx2-Q22) or mutant ataxin-2 forms (Atx2Q58 and Atx2Q104) and after 48 hours, these cells were collected for autophagic markers analysis by western blot (**A**). Through western blot analysis it is possible to verify an increase in SQSTM1 levels (**B**) and a significant decrease in LC3B-II levels (**C**) in cells transfected with mutant forms ataxin-2 compared to cells transfected with human wild-type ataxin-2 and non-transfected cells. [Data are expressed relative to β-actin levels ($n = 3$ independent experiments); Values are expressed as mean ± SEM; $^{*}P < 0.05$, $^{**}P < 0.01$; unpaired Student's t-test; one-way ANOVA followed by post hoc Tukey's multiple comparison test].

If there is no dysregulation of autophagy and considering that SQSTM1 is responsible for recognizing ubiquitinated substrates and directing them to

autophagosomes, the levels of this protein are decreased. In addition, LC3B-II is increased, because during the autophagic pathway this protein is necessary for the formation of the autophagosome, being obtained through the cleavage of Atg4 and subsequent binding to PE. Therefore, if impairment of autophagy occurs, as is expected in SCA2 disease, and as occurs with other polyQ diseases, SQSTM1 levels should be increased while LC3B-II levels should be decreased (Jimenez-Sanchez et al., 2012, Lee and Lee, 2016, Katsuragi et al., 2015).

So, these results suggest that there is a dysregulation of the autophagic pathway in cells that express mutant ataxin-2, contrary to what happens with non-transfected cells and cells transfected with Atx2Q22.

4.2. Detection of autophagic markers in brain tissue of SCA2 patients

Although there are already studies demonstrating the presence of autophagic markers and the accumulation of autophagosomes in the neuronal tissue of patients with SCAs, there is still no evidence of impairment of autophagy in the brain tissue of patients with SCA2 disease (Nascimento-Ferreira et al., 2011, Alves et al., 2014).

Therefore, immunohistochemical analyzes were performed to confirm the presence of autophagic markers in the brain of patients with SCA2. Through Figure 15D we confirm the presence of ataxin-2 aggregates in the cerebellum of SCA2 patients, as expected, since these are indicative of disease, contrary to what happens with samples from healthy individuals (Figure 15A).We also verified, through the figure, the presence of autophagic markers, such as LC3B (Figure 15E) and SQSTM1 (Figure 15F) in the cerebellum of patients with SCA2, while in healthy individuals the presence of these markers is not detected (Figure 15B, C).

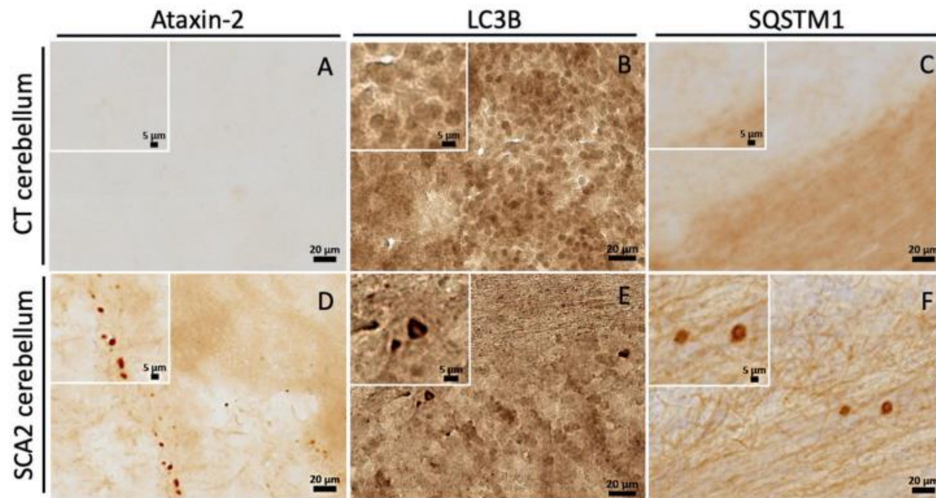

Figure 15: Images of cerebellum post-mortem brain sections of SCA2 patients and healthy controls by immunohistochemical analysis. In samples from healthy individuals, staining for ataxin-2 showed no immunoreactivity, so there is no presence of ataxin-2 aggregates (**A**), while in cerebellum tissue of SCA2 patients the presence of SCA2 aggregates is verified, as expected, since these aggregates are indicative of disease (**D**). In cerebellum tissue of SCA2 patients, staining for autophagic markers, LC3B and SQSTM1 (**E, F**), showed an abnormal accumulation of these proteins, while in samples from healthy individuals the same did not occur (**B, C**).

These alterations mentioned above and verified in Figure 15, also seem to be observed in the striatum of SCA2 patients (Figure 16). Immunohistochemical analysis of post-mortem brain tissue showed that in the striatum from patients with SCA2, there is the presence, as expected, of ataxin-2 aggregates and autophagic markers, SQSTM1 and LC3B (Figure 16D, E, F). However, in the striatum of healthy individuals this abnormal accumulation of ataxin-2 aggregates and autophagic markers is not observed (Figure 16A, B, C).

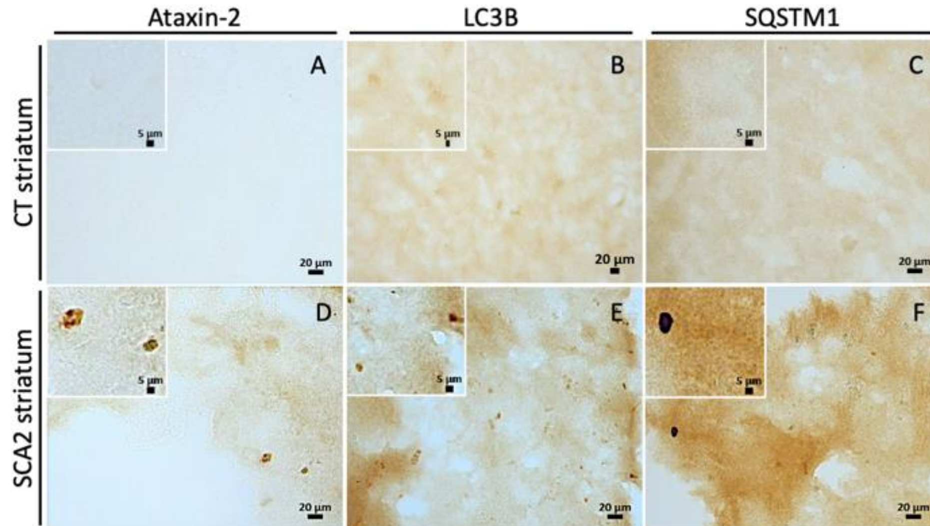

Figure 16: Images of striatum post-mortem brain sections of SCA2 patients and healthy controls by immunohistochemical analysis. In samples from healthy individuals, staining for ataxin-2 showed no immunoreactivity, so there is no presence of ataxin-2 aggregates (**A**), while in striatum tissue of SCA2 patients the presence of SCA2 aggregates is verified, as expected, since these aggregates are indicative of disease (**D**). In striatum tissue of SCA2 patients, staining for autophagic markers, LC3B and SQSTM1 (**E, F**), showed an abnormal accumulation of these proteins, while in samples from healthy individuals the same did not occur (**B, C**).

Based on these findings, it is possible to demonstrate for the first time that an autophagy dysfunction occurs in the brain of SCA2 patients.

4.3. Impact of cordycepin administration in the SCA2 striatal mouse model

In several polyQ diseases, such as SCA2, one of the pathogenesis mechanisms is the formation of aggregates due the expansion of CAG repeats. Thus, a possible future therapeutic option can be the reduction of these aggregates, through the induction of the autophagic pathway (Magaña et al., 2013).

A recent study, in which cordycepin treatment was used to induce autophagy and thus reduce the number of aggregates, was performed in SCA3 disease models. The

results of this study showed that cordycepin reduced ataxin-3 levels, the number of ubiquitin aggregates and motor and neuropathological deficits (Marcelo et al., 2019).

So, the aim was to demonstrate that the administration of cordycepin can rescue neuropathological features of SCA2 striatal mouse model and thus being able to become a possible future treatment of polyQ diseases.

To test this hypothesis, intraperitoneal injections of cordycepin (20mg/kg) and saline vehicle (control animals) were performed, during twelve weeks, in mice expressing mutant ataxin-2. After twelve weeks the mice were sacrificed and the impact of cordycepin treatment on their brain was analyzed (Figure 17A, B).

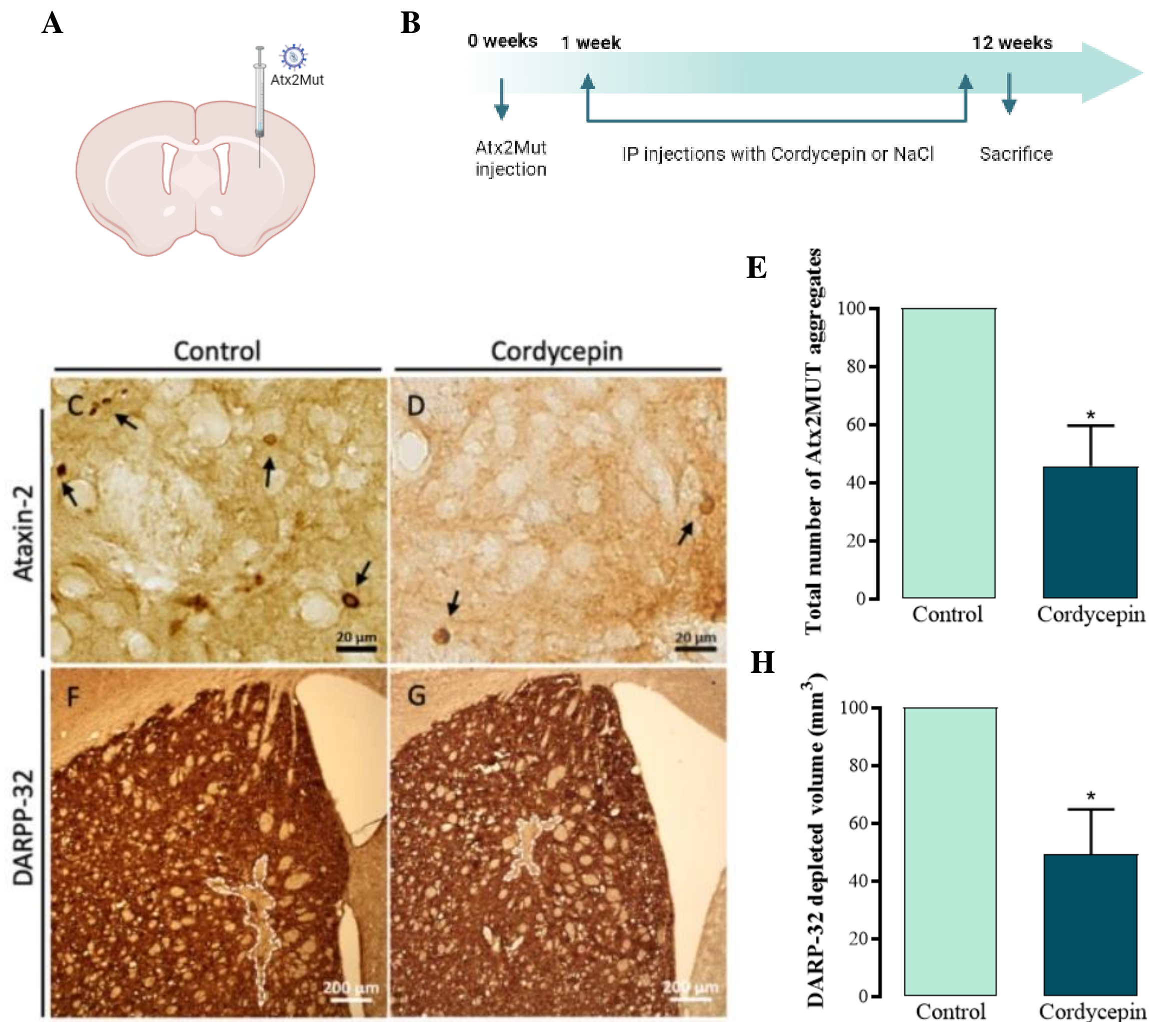

Figure 17: Administration of cordycepin resulted in a reduction in the presence of ataxin-2 aggregates and a decrease in the neuronal marker in SCA2 striatal mouse model. Lentivirus encoding for mutant ataxin-2 were administered

through a stereotaxic surgery into the right striatum hemisphere and then, for twelve weeks, these mice received either Cordycepin (20mg/Kg) or saline vehicle (NaCl 0,1%) IP injections (**A, B**). After 12 weeks the mice were sacrificed and through immunohistochemical analysis we can see that in the mice that received the IP injection of cordycepin there is a reduction in the total number of mutant ataxin-2 aggregates, compared (**C, D, E**). In samples from mice that received cordycepin injections, staining for DARP-32 showed a reduction in the depleted compared to the mice that received only saline vehicle group (**F, G, H**). (Values are expressed as mean ± SEM n = 3 (control) and n = 4 (cordycepin) relative to control group mean; *P < 0.05; unpaired Student's t-test).

The results show that animals injected with cordycepin have a significant reduction of the number of ataxin-2 aggregates, compared to non-treated animals injected only with saline vehicle (Figure 17C, D, E). In addition, animals injected with cordycepin also show a significant decrease of DARP-32, a neuronal marker staining volume, compared to non-treated animals (figure 17F, G, H).

Thus, we can verify that the pharmacologic activation of autophagy by cordycepin had a neuroprotective effect and lead to a reduction of neuropathological symptoms, such as the reduction of protein aggregates and preservation of the neuronal loss in the SCA2 striatal mouse model.

5. Conclusion and future perspectives

The aim of this project was to study autophagy pathway in SCA2 disease, through investigating autophagy markers, SQSTM1 and LC3B, in cellular and animal models for this disease and in *post-mortem* brain tissue from SCA2 patients. Besides that, we intended to investigate if the induction of autophagy by cordycepin could be used as a therapeutic strategy for this disease.

The results of the present study showed evidence of a dysfunction of the autophagic pathway in Neuro-2A cells transfected with mutant forms of ataxin-2 (there was an increase of SQSTM1 levels and a decrease of LC3B-II levels) and in the brain of SCA2 patients (images of cerebellum and striatum samples showed an increase of ataxin-2 aggregates and an abnormal accumulation of autophagic markers). Furthermore, the present study also demonstrates that administration of cordycepin leads to reduced ataxin-2 aggregation and decreased of neuronal loss in the SCA2 striatal mouse model.

In the future, it would be important to evaluate other autophagic markers, such as beclin-1, since autophagy is a dynamic process. In addition, the degradation of protein aggregates can also be regulated by UPS, so it is important to understand if it is also dysfunctional and if the upregulation of this pathway could be a possible therapeutic target for SCA2. Furthermore, it would be relevant, through behavior analysis, to verify the reduction of physical symptoms in SCA2 mice treated with cordycepin and compare the results with untreated mice.

Thus, with this study we were able to demonstrate the dysfunction of the autophagy pathway in cellular and in post-mortem brain tissue of SCA2 patients. Moreover, it was demonstrated that upregulation of autophagy by cordycepin can be used as a future therapeutic strategy for the treatment of SCA2 disease.

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7. Appendices

7.1. Appendix I - Measurements of weight and concentration/volume to be administered to mice of cordycepin and saline vehicle

		Peso											
		25/mar	01/abr	08/abr	15/abr	22/abr/19	29/abr/19	06/mai/19	13/mai/19	20/mai/19	27/mai/19	03/jun/19	11/jun/19
Gaiola 103	F1 (0 marcas)	20,7	22,05	21,5	22,2	21,04	21,6	22,1	21,65	22,4	22,3	22,5	21,8
	F2 (1 marca)	23	22,8	22,8	23,7	24	23,01	23,8	23,3	24,12	23,6	23,7	24,01
Gaiola 104	F1 (0 marcas)	23,14	23,7	23,2	23	23,6	23,9	24,4	23,8	24,6	23,6	23,4	23,43
	F2 (1 marca)	22,3	23	23,7	23,2	23,45	23,9	24,6	24,1	24,05	24,1	23,8	22,9
Gaiola 105	M1 (0 marcas)	27,7	27,9	28,1	28	28,44	28,3	28,35	28,3	28,2	28,24	27,9	28,14
	M2 (1 marca)	28,2	28,3	28,6	28,2	28,69	28,6	28,2	28,3	28,8	29,3	28,6	27,54

		Injecções (20mg cordy/kg)																				
		25/mar			28/mar			01/abr			04/abr			08/abr			11/abr			15/abr		
		Mg	Vol. (ul)	Vol. inj. (ul)	Mg	Vol. (ul)	Vol. inj. (ul)	Mg	Vol. (ul)	Vol. inj. (ul)	Mg	Vol. (ul)	Vol. inj. (ul)	Mg	Vol. (ul)	Vol. inj. (ul)	Mg	Vol. (ul)	Vol. inj. (ul)	Mg	Vol. (ul)	Vol. inj. (ul)
Gaiola 103	F1 (0 marcas)	0,414	160	160	0,414	82,8	80	0,414	82,8	80	0,441	88,2	90	0,441	88,2	90	0,43	86	90	0,43	86	90
(Cordy)	F2 (1 marca)	0,460	160	160	0,46	92	90	0,46	92	90	0,456	91,2	90	0,456	91,2	90	0,456	91,2	90	0,456	91,2	90
Gaiola 104	F1 (0 marcas)	0,463	160	160	0,4628	92,56	90	0,4628	92,56	90	0,474	94,8	90	0,474	94,8	90	0,464	92,8	90	0,464	92,8	90
(NaCl)	F2 (1 marca)	0,446	160	160	0,446	89,2	90	0,446	89,2	90	0,46	92	90	0,46	92	90	0,474	94,8	90	0,474	94,8	90
Gaiola 105	M1 (0 marcas)	0,554	160	160	0,554	110,8	110	0,554	110,8	110	0,558	111,6	110	0,558	111,6	110	0,562	112,4	110	0,562	112,4	110
(Cordy)	M2 (1 marca)	0,564	160	160	0,564	112,8	110	0,564	112,8	110	0,566	113,2	110	0,566	113,2	110	0,572	114,4	110	0,572	114,4	110

	18/abr			22/abr			25/abr			29/abr			30/abr			03/mai			06/mai			08/mai		
	Mg	Vol. (ul)	Vol. inj. (ul)	Mg	Vol. (ul)	Vol. inj. (ul)	Mg	Vol. (ul)	Vol. inj. (ul)	Mg	Vol. (ul)	/ol. inj. (ul	Mg	Vol. (ul)	/ol. inj. (ul	Mg	Vol. (ul)	/ol. inj. (ul	Mg	Vol. (ul)	/ol. inj. (ul	Mg	Vol. (ul)	/ol. inj. (ul
	0,444	88,8	90	0,444	88,8	90	0,4208	84,16	90	0,4208	84,16	90	0,432	86,4	90	0,432	86,4	90	0,432	86,4	90	0,442	88,4	90
	0,474	94,8	90	0,474	94,8	90	0,48	96	90	0,48	96	90	0,4602	92,04	90	0,4602	92,04	90	0,4602	92,04	90	0,476	95,2	90
	0,46	92	90	0,46	92	90	0,472	94,4	90	0,472	94,4	90	0,478	95,6	90	0,478	95,6	90	0,478	95,6	90	0,488	97,6	90
	0,464	92,8	90	0,464	92,8	90	0,469	93,8	90	0,469	93,8	90	0,478	95,6	90	0,478	95,6	90	0,478	95,6	90	0,492	98,4	90
	0,56	112	110	0,56	112	110	0,5688	113,76	110	0,5688	113,76	110	0,566	113,2	110	0,566	113,2	110	0,566	113,2	110	0,567	113,4	110
	0,564	112,8	110	0,564	112,8	110	0,5738	114,76	110	0,5738	114,76	110	0,572	114,4	110	0,572	114,4	110	0,572	114,4	110	0,564	112,8	110

10/mai			13/mai			15/mai			17/mai			20/mai			22/mai			24/mai			27/mai		
Mg	Vol. (ul)	Vol. inj. (ul	Mg	Vol. (ul)	Vol. inj. (ul	Mg	Vol. (ul)	Vol. inj. (ul	Mg	Vol. (ul)	Vol. inj. (ul	Mg	Vol. (ul)	Vol. inj. (ul	Mg	Vol. (ul)	Vol. inj. (ul	Mg	Vol. (ul)	Vol. inj. (ul	Mg	Vol. (ul)	Vol. inj. (ul
0,442	88,4	90	0,442	88,4	90	0,433	86,6	90	0,433	86,6	90	0,433	86,6	90	0,448	89,6	90	0,448	89,6	90	0,448	89,6	90
0,476	95,2	100	0,476	95,2	100	0,466	93,2	90	0,466	93,2	90	0,466	93,2	90	0,4824	96,48	100	0,4824	96,48	100	0,4824	96,48	100
0,488	97,6	100	0,488	97,6	100	0,476	95,2	100	0,476	95,2	100	0,476	95,2	100	0,492	98,4	100	0,492	98,4	100	0,492	98,4	100
0,492	98,4	100	0,492	98,4	100	0,482	96,4	100	0,482	96,4	100	0,482	96,4	100	0,481	96,2	100	0,481	96,2	100	0,481	96,2	100
0,567	113,4	110	0,567	113,4	110	0,566	113,2	110	0,566	113,2	110	0,566	113,2	110	0,564	112,8	110	0,564	112,8	110	0,564	112,8	110
0,564	112,8	110	0,564	112,8	110	0,566	113,2	110	0,566	113,2	110	0,566	113,2	110	0,576	115,2	110	0,576	115,2	110	0,576	115,2	110

28/mai			29/mai			30/mai			31/mai			03/jun			04/jun			05/jun			06/jun		
Mg	Vol. (ul)	Vol. inj. (ul	Mg	Vol. (ul)	Vol. inj. (ul	Mg	Vol. (ul)	Vol. inj. (ul	Mg	Vol. (ul)	Vol. inj. (ul	Mg	Vol. (ul)	Vol. inj. (ul	Mg	Vol. (ul)	Vol. inj. (ul	Mg	Vol. (ul)	Vol. inj. (ul	Mg	Vol. (ul)	Vol. inj. (ul
0,446	89,2	90	0,446	89,2	90	0,446	89,2	90	0,446	89,2	90	0,446	89,2	90	0,45	90	90	0,45	90	90	0,45	90	90
0,472	94,4	100	0,472	94,4	90	0,472	94,4	90	0,472	94,4	90	0,472	94,4	90	0,474	94,8	90	0,474	94,8	90	0,474	94,8	90
0,472	94,4	100	0,472	94,4	90	0,472	94,4	90	0,472	94,4	90	0,472	94,4	90	0,468	93,6	90	0,468	93,6	90	0,468	93,6	90
0,482	96,4	100	0,482	96,4	100	0,482	96,4	100	0,482	96,4	100	0,482	96,4	100	0,476	95,2	100	0,476	95,2	100	0,476	95,2	100
0,5648	112,96	110	0,5648	112,96	110	0,5648	112,96	110	0,5648	112,96	110	0,5648	112,96	110	0,558	111,6	110	0,558	111,6	110	0,558	111,6	110
0,586	117,2	110	0,586	117,2	120	0,586	117,2	120	0,586	117,2	120	0,586	117,2	120	0,572	114,4	110	0,572	114,4	110	0,572	114,4	110

07/jun			11/jun			12/jun			13/jun			14/jun			15/jun		
Mg	Vol. (ul)	Vol. inj. (ul	Mg	Vol. (ul)	Vol. inj. (ul	Mg	Vol. (ul)	Vol. inj. (ul	Mg	Vol. (ul)	Vol. inj. (ul	Mg	Vol. (ul)	Vol. inj. (ul	Mg	Vol. (ul)	Vol. inj. (ul
0,45	90	90	0,45	90	90	0,436	87,2	90	0,436	87,2	90	0,436	87,2	90	0,436	87,2	90
0,474	94,8	90	0,474	94,8	90	0,4802	96,04	100	0,4802	96,04	100	0,4802	96,04	100	0,4802	96,04	100
0,468	93,6	90	0,468	93,6	90	0,4686	93,72	90	0,4686	93,72	90	0,4686	93,72	90	0,4686	93,72	90
0,476	95,2	100	0,476	95,2	100	0,458	91,6	90	0,458	91,6	90	0,458	91,6	90	0,458	91,6	90
0,558	111,6	110	0,558	111,6	110	0,5628	112,56	110	0,5628	112,56	110	0,5628	112,56	110	0,5628	112,56	110
0,572	114,4	110	0,572	114,4	110	0,5508	110,16	110	0,5508	110,16	110	0,5508	110,16	110	0,5508	110,16	110