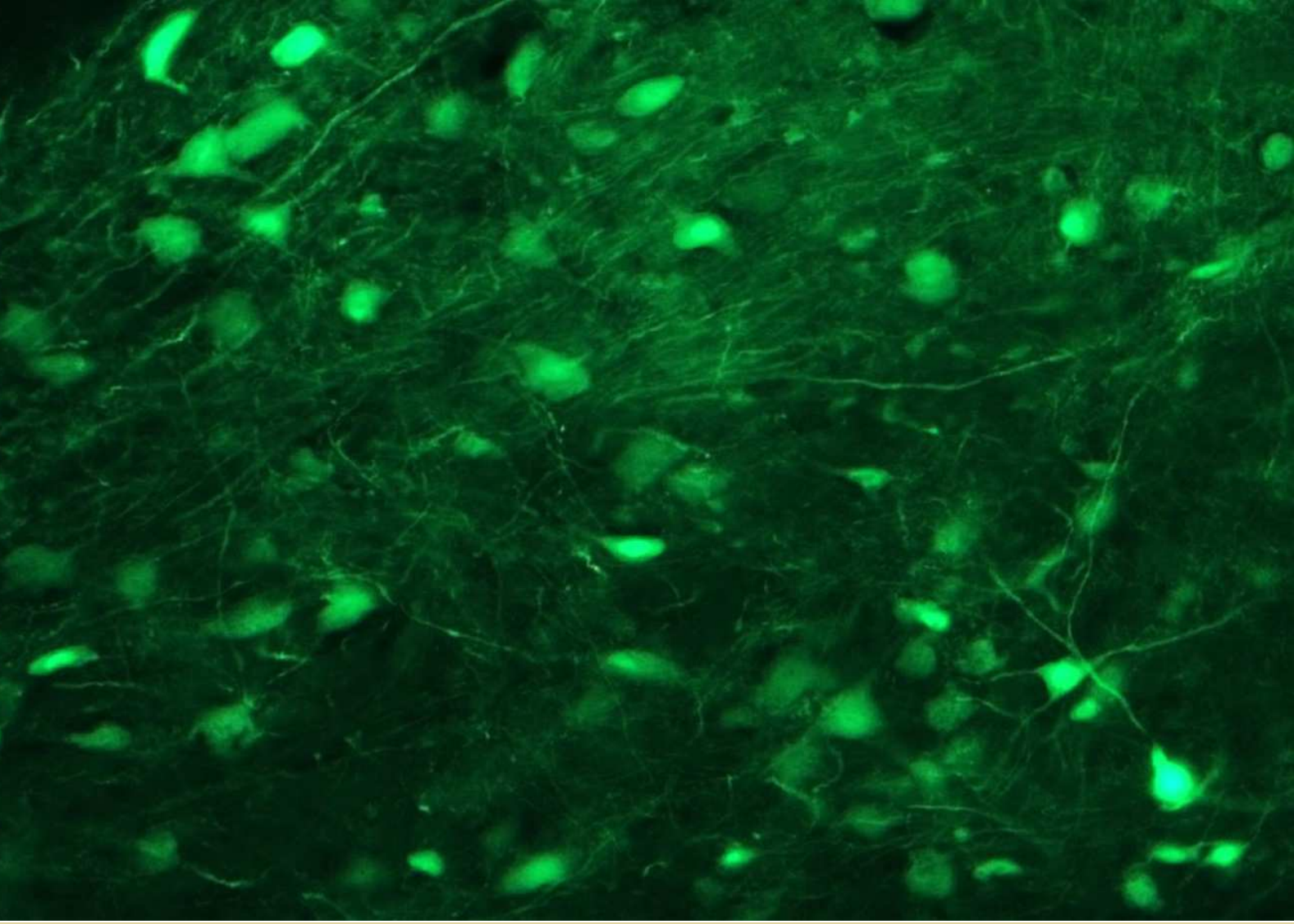

Joana Filipa Duarte das Neves

# NEUROPEPTIDE Y GENE TRANSFER FOR NEUROPROTECTION IN MACHADO-JOSEPH DISEASE

Tese de doutoramento em Ciências Farmacêuticas, na especialidade em Biotecnologia Farmacêutica, orientada pelo Professor Doutor Luís Pereira de Almeida e pela Professora Doutora Cláudia Cavadas e apresentada na Faculdade de Farmácia da Universidade de Coimbra

Setembro 2015

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Universidade de Coimbra

# Neuropeptide Y gene transfer for neuroprotection in Machado-Joseph disease

**Joana Filipa Duarte das Neves**

Thesis submitted to the Faculty of Pharmacy of the University of Coimbra for the attribution of the Doctor degree in Pharmaceutical Sciences, in the specialty field of Pharmaceutical Biotechnology.

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The research work presented in this thesis was performed at the Center for Neuroscience and Cell Biology, University of Coimbra and at the Faculty of Pharmacy of the University of Coimbra, Portugal, under the supervision of Professor Luís Pereira de Almeida and Professor Cláudia Cavadas.

O trabalho experimental apresentado nesta tese foi elaborado no Centro de Neurociências e Biologia Celular, Universidade de Coimbra e na Faculdade de Farmácia da Universidade de Coimbra, Portugal, sob a supervisão do Professor Luís Pereira de Almeida e da Professora Cláudia Cavadas.

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**Front cover:**

Microscope image of EGFP-expressing neurons in mouse cerebellum, after stereotaxic injection of AAV vectors encoding for EGFP.

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Esta tese é-vos dedicada.

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**Abbreviations**

| | |
|---|---|
| 6-OHDA | 6-hydroxydopamine |
| AAV | Adeno-associated virus |
| ACTH | Adrenocorticotropic hormone |
| AD | Alzheimer's disease |
| AgRP | Agouti-related protein |
| AmP | Aminopeptidase P |
| AMPA | Alpha-amino-3-hydroxyl-5-methyl-4-isoxazole propionic acid |
| ANOVA | Analysis of variance |
| Arc | Arcuate nucleus |
| Atx3 | Ataxin-3 |
| Atx3MUT | Mutant ataxin-3 |
| Atx3WT | Wild-type ataxin-3 |
| BDNF | Brain-derived neurotrophic factor |
| BrdU | 5-bromo-2'-deoxyuridine |
| BSA | Bovine serum albumin |
| CAG | Cytosine-adenine-guanine |
| CAMKII | Calmodulin-dependent protein kinase II |
| CART | Cocaine- and amphetamine-regulated transcript |
| cDNA | Complementary deoxyribonucleic acid |
| CMV | Cytomegalovirus |
| CNS | Central nervous system |
| CPB | Carboxypeptidase B |
| CPON | C-flanking peptide of NPY |
| CREB | cAMP response element-binding |
| CSF | Cerebrospinal fluid |
| Ct | threshold cycle |
| Cu/Zn SOD | Cu, Zn superoxide dismutase |
| DAB | 3,3'-diaminobenzidine tetrahydrochloride |
| DAPI | 4',6-diamino-2-phenylindole |
| DARPP-32 | Dopamine- and cyclic AMP-regulated phosphoprotein of 32 kDa |
| DNA | Deoxyribonucleic acid |
| DPP-IV | Dipeptidyl peptidase IV |
| DRPLA | Dentatorubral-pallidoluysian atrophy |
| DTT | Dithiothreitol |
| DUB | Deubiquitinating enzyme |
| EGFP | Enhanced green fluorescent protein |

| | |
|---|---|
| ER | Endoplasmic reticulum |
| GABA | Gamma-aminobutyric acid |
| GFAP | Glial fibrillary acidic protein |
| GL | Granular layer |
| HA | Hemagglutinin |
| HD | Huntington’s disease |
| HPA | Hypothalamic-pituitary-adrenal |
| Hsp | Heat shock protein |
| Iba1 | Ionized calcium-binding adapter molecule 1 |
| ICV | Intracerebroventricular |
| IHC | Immunohistochemistry |
| IL-1β | Interleukin-1 beta |
| IL-6 | Interleukin-6 |
| Ir | immunoreactivity |
| ISH | *In situ* hybridization |
| KO | Knock-out |
| LH | Lateral hypothalamus |
| LV | Lentivirus |
| MCH | Melanin concentrating hormone |
| MDMA | 3,4-methylenedioxymethamphetamine |
| miR | microRNA |
| MJD | Machado-Joseph disease |
| ML | Molecular layer |
| MPTP | 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine |
| mRNA | Messenger RNA |
| NES | Nuclear export signal |
| NGF | Nerve growth factor |
| NGS | Normal goat serum |
| NII | Neuronal intranuclear inclusion |
| NLS | Nuclear localization signal |
| METH | Methamphetamine |
| NMDA | N-methyl-D-aspartate |
| NPY | Neuropeptide Y |
| OD | Optical density |
| Orx | Orexin |
| PAM | Peptidylglycine alpha-amidating monooxygenase |
| PBS | Phosphate-buffered saline |
| PCR | Polymerase chain reaction |

| | |
|---|---|
| PD | Parkinson’s disease |
| PET | Positron emission tomography |
| PFA | Paraformaldehyde |
| PGK | Phosphoglicerate kinase 1 |
| PMSF | Phenylmethylsulphonyl fluoride |
| PolyQ | Polyglutamine |
| POMC | Pro-opiomelanocortin |
| PP | Pancreatic polypeptide |
| PYY | Peptide YY |
| QA | Quinolinic acid |
| RIA | Radioimmunoassay |
| RIPA | Radioimmunoprecipitation assay |
| RNA | Ribonucleic acid |
| ROS | Reactive oxygen species |
| RT | Room temperature |
| SBMA | Spinal and bulbar muscular atrophy |
| SCA | Spinocerebellar ataxia |
| SDS | Sodium dodecyl sulphate |
| SEM | Standard error of the mean |
| SGZ | Subgranular zone |
| shRNA | Short-hairpin RNA |
| SVZ | Subventricular zone |
| Tg | Transgenic |
| TMT | Trimethyltin |
| TNFα | Tumor necrosis factor-alpha |
| TrkB | Tropomyosin receptor kinase B |
| TUNEL | Terminal deoxynucleotidyl transferase dUTP nick end labeling |
| Ub | Ubiquitin |
| UIM | Ubiquitin-interacting motif |
| UPS | Ubiquitin-proteasome system |
| WT | Wild-type |
| YAC | Yeast artificial chromosome |

**Summary**

Machado-Joseph disease or spinocerebellar ataxia type 3 (MJD/SCA3) is the most common dominantly inherited ataxia worldwide. It is caused by an over-repetition of the trinucleotide CAG, which translates into an expanded polyglutamine tract within the protein ataxin-3. This mutated protein acquires toxic properties promoting neurodegeneration responsible for a progressive impairment of balance and motor function. There is currently no therapy to prevent or slow down the progression of this neurodegenerative disorder.

Neuropeptide Y (NPY) is an abundant peptide widely distributed through the mammalian brain, involved in several physiological functions. NPY demonstrated to be effective in controlling and alleviating neurodegeneration in different brain diseases, including Alzheimer's, Parkinson's and Huntington's disease. Therefore, we evaluated NPY levels in postmortem patient samples and mouse models. Moreover, we investigated whether NPY gene transfer would control MJD-associated neuropathology and motor behavior defects in MJD mouse models.

The hypothalamus is one of the main regulators of energy homeostasis, sleep and emotion. This brain region is affected by the pathogenic mechanisms of some neurodegenerative disorders, which has, in fact, been associated with some non-motor symptoms of Parkinson's and Huntington's disease patients. Considering that MJD patients present non-motor features, such as weight loss and sleep disturbances, we investigated the involvement of hypothalamus in MJD neuropathology, using MJD transgenic mice.

This thesis is organized in 5 chapters. In chapter 1, a review about MJD and NPY, with emphases in NPY neuroprotective properties, is presented.

In chapter 2, we evaluated striatal NPY levels in a lentiviral (LV)-based striatal MJD mouse model. Since NPY levels were reduced in mutant ataxin-3 transduced striata, we used this striatal MJD model to overexpress NPY mediated by adeno-associated viral (AAV) vectors. Our results show that NPY overexpression reduces the number of mutant ataxin-3 aggregates and decreases striatal pathology. Moreover, our data suggest that NPY-induced neuroprotection is associated with increased levels of BDNF and decreased levels of neuroinflammation markers.

In chapter 3, we investigated NPY levels in the cerebellum (the MJD most affected brain region) in post-mortem tissue of MJD patients and in cerebellar extracts of a MJD transgenic mouse model. We observed a decrease of NPY levels either in dentate nucleus of MJD patient samples and in transgenic mice cerebella. Considering the neuroprotective properties of NPY in the striatal mouse model, we tested NPY ability to alleviate MJD cerebellar neuropathology. For that, AAV vectors encoding NPY were injected centrally in the cerebellum of transgenic mice, which then undergone a series of behavioral tests to evaluate motor paradigms. We provide evidence that NPY overexpression prevents balance

and motor impairments associated with an attenuation of the underlying cerebellar morphological alterations.

In chapter 4, two transgenic mouse models of MJD were used to explore the potential involvement of hypothalamus in MJD neuropathology. Immunohistochemical analysis revealed a decreased number of Orexin (Orx)-, melanin-concentrating hormone (MCH)- and pro-opiomelanocortin (POMC)-positive neurons in the hypothalamus of MJD transgenic mice that present an accumulation of mutant ataxin-3 in these hypothalamic neurons (CAMKII MJD mice). In fact, no such hypothalamic alterations were observed in MJD transgenic mice that only express the mutated protein in cerebellar Purkinje cells (L7 MJD mice).

Finally, in chapter 5, final remarks and future perspectives are presented. In conclusion, this thesis provides evidence that NPY levels are decreased in the cerebellum of MJD patients and in the cerebellum and striatum of two well-characterized MJD mouse models. Moreover, we show that NPY overexpression mediates neuroprotection in those MJD mice, which thus maybe an effective therapeutic strategy for MJD. Furthermore, this study indicates that the hypothalamus is affected in a transgenic MJD mouse model in which mutant ataxin-3 is accumulated in hypothalamic neurons. This way, we suggest the establishment of hypothalamus as a new therapeutic target in MJD.

**Resumo**

A doença de Machado-Joseph ou ataxia espinocerebelosa tipo 3 (MJD/SCA3) é a ataxia autossómica dominante mais comum a nível mundial. É causada por uma repetição excessiva do trinucleótido CAG, que se traduz numa expansão da cadeia de poliglutaminas na proteína ataxina-3. Esta proteína mutada adquire propriedades tóxicas e promove neurodegenerescência responsável pela progressiva disfunção da coordenação motora e do equilíbrio. Não existe atualmente nenhuma terapêutica que permita prevenir ou atrasar a progressão desta doença neurodegenerativa.

O Neuropeptídeo Y (NPY) é um peptídeo abundante e amplamente distribuído pelo cérebro de mamíferos, estando envolvido em diversas funções fisiológicas. Múltiplas evidências mostram a capacidade do NPY para controlar e mitigar a neurodegenerescência em diferentes doenças neurológicas, tais como a doença de Alzheimer, a doença de Parkinson e a doença de Huntington. Assim, avaliámos os níveis de NPY em amostras de doentes MJD e de modelos de murganhos da doença e testámos a capacidade do NPY para atenuar a neuropatologia associada à MJD nestes modelos animais.

O hipotálamo é um dos principais reguladores da homeostase energética, do sono e das emoções. Esta região cerebral é afetada pelos mecanismos patogénicos de algumas doenças neurodegenerativas, o que foi já relacionado com a ocorrência de alguns sintomas não motores nos doentes de Parkinson e de Huntington. Tendo em conta que os doentes MJD também apresentam sintomas não motores, como perda de peso corporal e perturbações do sono, investigámos o envolvimento do hipotálamo na neuropatologia da MJD, usando murganhos transgénicos da doença.

A tese encontra-se organizada em 5 capítulos. No capítulo 1, é apresentado um resumo bibliográfico da MJD e do NPY, com ênfase nas suas propriedades neuroprotectoras.

No capítulo 2, avaliámos os níveis estriatais de NPY no modelo lentiviral estriatal de murganho da MJD. Uma vez que os níveis de NPY estão diminuídos nos hemisférios estriatais transduzidos com a ataxina-3 mutada, utilizámos este modelo animal para promover a sobreexpressão do NPY, mediada por vectores virais adeno-associados (AAV). Os nossos resultados mostram que a sobreexpressão de NPY reduz o número de agregados de ataxin-3 mutante e diminui a patologia estriatal. Simultaneamente, os resultados sugerem que esta neuroprotecção induzida pelo NPY está associada ao aumento dos níveis de BDNF e à diminuição dos níveis de marcadores de neuroinflamação.

No capítulo 3, avaliámos os níveis de NPY no cerebelo (a região do sistema nervoso central mais afetada pela MJD) em amostras *postmortem* de doentes MJD e em extratos cerebelares de murganhos transgénicos da doença. Observamos uma diminuição dos níveis de NPY no cerebelo dos doentes e dos animais transgénicos MJD. Tendo em conta os

efeitos neuroprotectores do NPY no modelo estriatal da MJD, testámos a capacidade do NPY para diminuir a neuropatologia cerebelar associada à doença. Para isso, vectores AAV que codificam para o NPY foram injetados centralmente no cerebelo dos murganhos transgénicos, que foram depois sujeitos a uma série de testes comportamentais para avaliar a sua função motora. Evidenciamos que a sobreexpressão de NPY previne défices motores e de equilíbrio, atenuando também as alterações morfológicas cerebelares associadas à ataxia.

No capítulo 4, dois modelos transgénicos da MJD foram utilizados para explorar o potencial envolvimento do hipotálamo na neuropatologia da doença. Análises imunohistoquímicas revelam uma diminuição do número de neurónios que expressam orexina (Orx), hormona concentradora de melanina (MCH) e pro-opiomelanocortina (POMC) no hipotálamo de animais transgénicos que apresentam acumulação de ataxina-3 mutada nos neurónios desta zona cerebral (murganhos CAMKII MJD). Em conformidade, estas alterações hipotalâmicas não se observam nos animais transgénicos que apenas expressam a proteína mutada nas células de Purkinje do cerebelo (murganhos L7 MJD).

Por último, no capítulo 5, são apresentadas considerações finais e perspetivas futuras. Concluindo, esta tese revela que os níveis de NPY no cerebelo de doentes MJD e no cerebelo e estriado de dois modelos roedores bem caracterizados da doença estão diminuídos. Evidencia também que a sobreexpressão do NPY promove neuroprotecção nestes mesmos modelos MJD, sugerindo que poderá assim vir a constituir uma estratégia terapêutica eficaz para a MJD. Por fim, este estudo indica que o hipotálamo é afetado pela MJD, como avaliado em animais que apresentam acumulação de ataxina-3 mutante nos neurónios hipotalâmicos. Desta forma, sugerimos que o hipotálamo seja instituído como um novo alvo terapêutico na MJD.

# CHAPTER 1

# General introduction

## 1.1. Neurodegenerative disorders

The prevalence of neurodegenerative diseases is increasing with the expansion of life span that takes place particularly in developing countries (Global Burden of Disease Study, 2015). These disorders are usually characterized by a late onset and involvement of selective neuronal dysfunction or loss, and due to their progressive course they impose substantial medical and public health burdens (Checkoway *et al.*, 2011). Despite progress has been made in unravelling their pathogenic mechanisms, it is still lacking appropriate therapeutic strategies to allow prevention or arrest of disease progression.

Alzheimer's disease (AD) is the most frequent type of dementia that occurs in the middle to late life (Qiu *et al.*, 2009). The course of the disorder usually begins with amnesia and proceeds to effects on language and motor skills, revealing a progressive loss of cognitive function (Folstein and Whitehouse, 1983), and its neuropathology is characterized by the presence of neuritic plaques (aggregates of amyloid β), tau neurofibrillary tangles and specific neuronal loss (McKhann *et al.*, 1984), that primarily affects hippocampus and neocortex (Morrison and Hof, 1997).

Parkinson's disease (PD) is the second most common neurodegenerative disorder after AD. The genetic mutations discovered until now (Hardy *et al.*, 2003) likely only explain a small proportion of all PD – about 90% of cases are apparently idiopathic and thought to result from complex interactions between environment and genetic factors (de Lau and Breteler, 2006). PD is characterized by a progressive degeneration of the dopaminergic nigrostriatal system associated with widespread Lewy bodies (mainly constituted by α-synuclein) (Jellinger, 1991), which is responsible for PD's clinical motor features: resting tremor, bradykinesia and rigidity (Hornykiewicz, 1975). Furthermore, around 40% of PD patients eventually develop dementia due to spread of degeneration and Lewy bodies to the cerebral cortex and limbic structures (Emre, 2003).

Other important category of these central nervous system-affecting disorders is the group of polyglutamine diseases, which comprise one of the most common groups of inherited neurodegenerative conditions.

### 1.1.1. Polyglutamine diseases

Polyglutamine (polyQ) diseases consist of at least nine diseases caused by the pathological expansion of trinucleotide CAG repeats that encode an elongated polyQ tract in the mutated proteins, which due to conformational changes tend to aggregate (Figure 1.1). The first polyQ disease identified was spinal and bulbar muscular atrophy (SBMA) (La Spada *et al.*, 1991); Huntington's disease (HD), dentatorubral-pallidoluysian atrophy (DPRLA) and

spinocerebellar ataxias (SCA) types 1, 2, 3, 6, 7 and 17 were later added to this class of neurodegenerative disorders (Table 1.1).

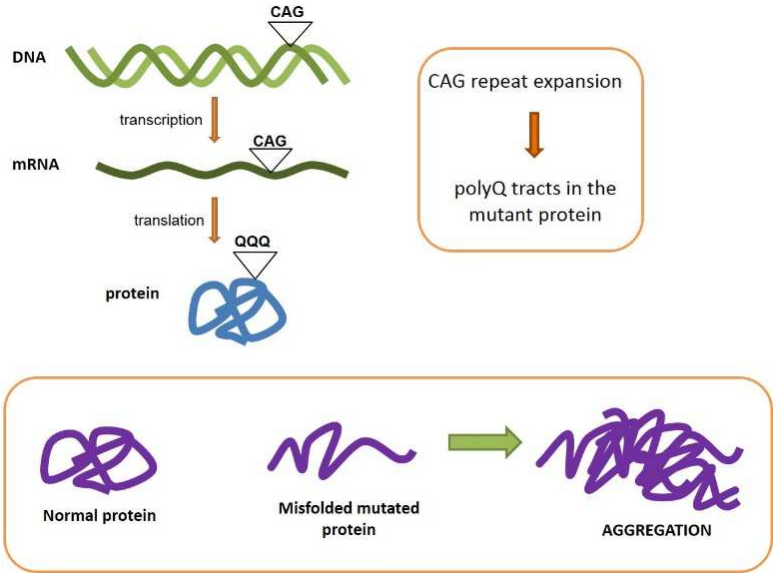


**Figure 1.1 – Schematic representation of PolyQ proteins formation and aggregation.** An over-repetition of the CAG trinucleotide translates into an expanded polyQ stretch in the mutated protein, which due to conformational changes tends to aggregate.

**Table 1.1 – Polyglutamine diseases**

| PolyQ disease | Protein | Normal subcellular localization | CAG repeats | | Most affected brain regions |
|---|---|---|---|---|---|
| | | | Healthy | Disease | |
| SBMA | Androgen receptor | Nucleus and cytoplasm | 9-36 | 38-62 | Anterior horn and bulbar neurons, dorsal root ganglia |
| HD | Huntingtin | Cytoplasm | 6-34 | 36-121 | Striatum and cerebral cortex |
| DRPLA | Atrophin-1 | Cytoplasm | 7-34 | 49-88 | Cerebellum, cerebral cortex, basal ganglia, Luys body |
| SCA1 | Ataxin-1 | Nucleus | 6-39 | 40-82 | Purkinje cells, dentate nucleus, brainstem |
| SCA2 | Ataxin-2 | Cytoplasm | 15-24 | 32-200 | Purkinje cells, brainstem, fronto-temporal lobes |
| SCA3 / MJD | Ataxin-3 | Cytoplasm | 12-44 | 61-87 | Dentate nucleus, basal ganglia, brainstem, spinal cord |
| SCA6 | $\alpha_{1A}$- voltage-dependent calcium channel subunit | Cell membrane | 4-20 | 20-29 | Purkinje cells, dentate nucleus, inferior olive |
| SCA7 | Ataxin-7 | Nucleus | 4-35 | 37-306 | Cerebellum, brainstem, macula, visual cortex |
| SCA17 | TATA-binding protein (TBP) | Nucleus | 25-42 | 47-63 | Purkinje cells, inferior olive |

The nine known polyQ diseases are listed along with their causative proteins, their normal subcellular localization, the number of CAG repeats that cause disease and brain regions most

affected. Table was adapted from (Cummings and Zoghbi, 2000, Paulson *et al.*, 2000, Zoghbi and Orr, 2000, Shao and Diamond, 2007).

The neurodegeneration may depend directly on the polyQ expansions, since the mutated proteins responsible for this disorders do not share any homology in structure and do not present similar functions (Cummings and Zoghbi, 2000). Furthermore, it was shown in cellular models that the expression of an expanded polyQ tract alone induces cell death and, in animal models that polyQ causes motor impairments and premature death (Ikeda *et al.*, 1996, Ordway *et al.*, 1997, Senut *et al.*, 2000). Moreover, disease severity is positively correlated with the length of polyQ tract (Maciel *et al.*, 1995, Ikeda *et al.*, 1996).

However, the context of the "host" protein is likely responsible for the clinical differences of the distinct diseases (Orr and Zoghbi, 2007) and for the variation of the absolute number of repeats necessary to cause each disease. Furthermore, although most of the mutated proteins are expressed throughout the CNS, in each disorder only certain neuronal populations are susceptible to their toxicity (Duenas *et al.*, 2006, Bauer and Nukina, 2009). This may be due to selective protein interactions driven by the unique properties of the pathogenic protein (Taroni and DiDonato, 2004).

In general, early ages at onset of the disease are associated with higher CAG repeats (Maciel *et al.*, 1995, Ranum *et al.*, 1995, van de Warrenburg *et al.*, 2002). The expanded CAG repeat is unstable and tends to further expand, leading to an early age at onset and higher severity of disease in the successive generations – this phenomenon is called anticipation (Maciel *et al.*, 1995, Durr *et al.*, 1996, Paulson, 2007).

Among polyQ diseases, Huntington's disease (HD) has the highest prevalence. HD is characterized by a progressive physical and mental incapacitation (The Huntington's Disease Collaborative Research, 1993). The mutated huntingtin, that forms intranuclear aggregates, leads to severe atrophy of the caudate nucleus and putamen (Mann *et al.*, 1993), preferentially affecting projection neurons. This neurodegeneration is accountable for the clinical manifestations of the disease: progressive motor disability with chorea or rigidity, cognitive impairment and emotional disturbance (Vonsattel and DiFiglia, 1998).

#### 1.1.1.1. Machado-Joseph disease (MJD)

Machado-Joseph disease (MJD), or spinocerebellar ataxia type 3 (SCA3), was first identified in Northern American families of Azorean ancestry. Initially, the disease was reported as four distinct entities: Machado Disease (Nakano *et al.*, 1972), Nigro-spino-dentatal degeneration (Woods and Schaumburg, 1972), Joseph disease (Rosenberg *et al.*, 1976) and Azorean disease of the nervous system (Romanul *et al.*, 1977). However, Countinho and Andrade later proposed that those diseases were in fact simply variations of

a single genetic disorder which they named Machado-Joseph disease (Coutinho and Andrade, 1978).

Some authors suggested that MJD was originated in Portugal and disseminated worldwide by the Portuguese navigators in the 16th century (Sequeiros and Coutinho, 1993, Rosenberg, 1995, Gaspar *et al.*, 2001). However, the presence of the disease in various countries make this hypothesis virtually impossible (Ranum *et al.*, 1995, Lima *et al.*, 1998).

#### 1.1.1.1.1. Prevalence

MJD is a rare neurodegenerative disorder. In fact, all SCAs together have a prevalence of 2.7/100000 (Ruano *et al.*, 2014). Despite of that, MJD is the most common dominantly inherited ataxia worldwide (21%), being more prevalent in Portugal and Brazil (Schöls *et al.*, 2004). Actually, the highest prevalence reported worldwide occurs in Flores Island in Azores, Portugal (1/239), where it represents an important public health problem (Bettencourt *et al.*, 2008).

#### 1.1.1.1.2. Neuropathology and clinical symptoms

In MJD, neuronal loss occurs in selective regions of the CNS: cerebellum (dentate nucleus and spinocerebellar pathways), brainstem (pontine and cranial nerves nuclei), spinal cord (anterior horns and Clarke's columns), basal ganglia (*substantia nigra*, striatum and *globus pallidus*), thalamus and cerebral cortex (Rosenberg *et al.*, 1976, Coutinho *et al.*, 1982, Sudarsky and Coutinho, 1995, Klockgether *et al.*, 1998, Rub *et al.*, 2003, Rub *et al.*, 2005, Rub *et al.*, 2006b, Alves *et al.*, 2008b, de Rezende *et al.*, 2015). Positron emission tomography (PET) scans showed a decreased regional cerebral glucose metabolism in these brain regions (Taniwaki *et al.*, 1997, Wullner *et al.*, 2005), and that this changes occur even before the onset of clinical symptoms in MJD gene carriers (Soong and Liu, 1998).

MJD is characterized by a wide range of clinical manifestations. Mainly, MJD patients present ataxia, which manifests in gait imbalance and limb incoordination, and also dystonia, Parkinson-like features of tremor-at-rest, bradykinesia and rigidity, ophthalmoparesis, nystagmus, facial and lingual fasciculations, bulging eyes due to eyelid retraction, dysphagia and dysarthria (Lima and Coutinho, 1980, Rosenberg, 1992, Sudarsky and Coutinho, 1995, Schöls *et al.*, 2004, Riess *et al.*, 2008, Raposo *et al.*, 2014). The motor impairments are progressive and eventually gait and limb ataxia becomes so severe that the patients need to use gait-assist devices, wheelchair and ultimately be bedridden (Paulson, 2007).

Furthermore, there are also non-motor features with significant prevalence in MJD patients: depressive symptoms, sleep disturbances, olfactory dysfunction, weight loss and metabolic changes (Cecchin *et al.*, 2007, Braga-Neto *et al.*, 2011, Pedroso *et al.*, 2011, Saute *et al.*, 2011, Saute *et al.*, 2012, Roeske *et al.*, 2013, dos Santos *et al.*, 2014). These manifestations can have a high impact in patients' quality of life, increasing the impairment in everyday functioning.

#### 1.1.1.1.3. MJD management

Until now there are no effective therapeutic strategies available to prevent, attenuate or stop disease progression. Therefore, management of MJD is based on symptoms alleviation: parkinsonism, bradykinesia and restless leg syndrome (one of the causes of sleep disturbances in these patients) may respond to amantadine, levodopa or dopaminergic agonists (Tuite *et al.*, 1995, Schols *et al.*, 1998, Buhmann *et al.*, 2003, Nandagopal, 2004, Paulson, 2007); spasticity respond to baclofen, tizanidine or memantine (Riess *et al.*, 2008); dystonia can be treated with local botulinum toxin injections (Freeman and Wszolek, 2005); sleep disturbances can be treated with benzodiazepines (Paulson, 2007); and depression may respond to antidepressants (D'Abreu *et al.*, 2010).

Moreover, non-pharmacological approaches such as physical and occupational therapy can make a significant practical difference in quality of life of MJD patients (Margolis, 2002, Paulson, 2007, D'Abreu *et al.*, 2010).

#### 1.1.1.1.4 Genetics

Considering the variability of clinical manifestations of MJD, the accurate diagnosis is made based on the detection of the genetic mutation (Cancel *et al.*, 1995, Maciel *et al.*, 1995). MJD is caused by a mutation in *MJD1*/*ATXN3* gene, in the long arm of chromosome 14, region 14q32.1 (Takiyama *et al.*, 1993, Kawaguchi *et al.*, 1994). This mutation consists in an expansion of CAG repeats: in healthy individuals the gene contains 12-44 CAG repeats whereas MJD patients show 61-87 CAG repeats (Maciel *et al.*, 2001). Intermediate size alleles are rare, but there are a few reports of disease caused by 45, 51, 53, 54, 55 and 56 CAG repeats (reviewed in (Bettencourt and Lima, 2011)).

#### 1.1.1.1.5. Ataxin-3

The MJD causing mutation results in an abnormally long polyQ tract at the C-terminus of ataxin-3 (Atx3) (Kawaguchi *et al.*, 1994, Masino *et al.*, 2003). The non-expanded Atx3 protein has a molecular weight of approximately 42 kDa, widely expressed throughout the body, in neuronal and non-neuronal tissues (Paulson *et al.*, 1997a, Paulson *et al.*, 1997b, Wang *et al.*, 1997, Schmidt *et al.*, 1998). Atx3 expression is more abundant in some neuronal populations than in others, although there is no relationship to the likelihood of neurons to undergo degeneration in the disease (Paulson *et al.*, 1997a, Schmidt *et al.*, 1998).

Atx3 contains a conserved N-terminal Josephin domain followed by two ubiquitin-interacting motifs (UIMs) and the polyQ tract (Masino *et al.*, 2003, Albrecht *et al.*, 2004, Chow *et al.*, 2004, Mao *et al.*, 2005). The protein has different isoforms due to alternative splicing (Bettencourt *et al.*, 2010), and the most common one expressed in the brain has a third UIM after the polyQ stretch (Harris *et al.*, 2010).

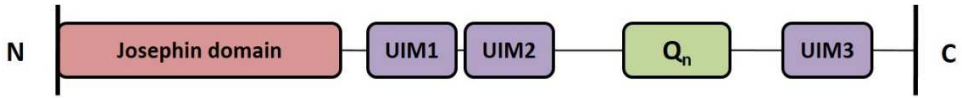


**Figure 1.2 – Diagram of ataxin-3 protein primary structure.** Ataxin-3 consists of an N-terminal Josephin domain, followed by a more flexible C-terminus that contains two or three UIMs and the polyQ stretch.

Atx3 is normally a predominantly cytoplasmic protein (Paulson *et al.*, 1997b, Schmidt *et al.*, 1998), but it is also present in the nucleus (Tait *et al.*, 1998, Trottier *et al.*, 1998, Fujigasaki *et al.*, 2000). However, when Atx3 has an expanded polyQ stretch it accumulates in the nucleus where it forms neuronal intranuclear inclusions (NIIs) in affected brain regions (Paulson *et al.*, 1997a, Paulson *et al.*, 1997b, Schmidt *et al.*, 1998). Actually, the polyQ tract expansion can lead to conformational changes preventing the proper folding of the protein promoting its aggregation (Masino *et al.*, 2003, Nagai *et al.*, 2007). Furthermore, NIIs sequestrate heat shock proteins (Hsp), such as Hsp40 and Hsp70 (Chai *et al.*, 1999a, Schmidt *et al.*, 2002). Considering that Hsp recognize misfolded proteins, stabilize and suppress their aggregation (Muchowski *et al.*, 2000), Hsp sequestration probably exacerbate inclusions formation. These NIIs constitute a hallmark of the disease. It has been suggested that the efficient and rapid development of cytotoxicity in response to polyQ expansion, both in *in vitro* and *in vivo* disease models, requires both their aggregation and their nuclear localization (Yang *et al.*, 2002, Bichelmeier *et al.*, 2007). However, the simple presence of mutant Atx3 aggregates in the nucleus is not sufficient to cause cell degeneration (Trottier *et al.*, 1998, Warrick *et al.*, 1998, Boy *et al.*, 2010, Silva-Fernandes *et al.*, 2010).

Furthermore, there is evidence of proteolytic cleavage of Atx3 (Tarlac and Storey, 2003, Goti *et al.*, 2004). Indeed, mutant Atx3 cleavage fragments were detected in brains of

MJD transgenic mice and in the affected brain regions of MJD patients (Goti *et al.*, 2004, Simoes *et al.*, 2012). These mutant Atx3 fragments lead to an increased formation of aggregates and enhanced neuronal degeneration, as evidenced in cellular (Berke *et al.*, 2004), fly (Jung *et al.*, 2009) and mouse models of MJD (Simoes *et al.*, 2012, Simoes *et al.*, 2014).

Multiple studies have tried to clarify the cellular and physiological roles of Atx3 (reviewed in (Costa and Paulson, 2012, Evers *et al.*, 2014). Several evidence suggest a role in regulation of protein degradation. In fact, the Josephin domain, that has an ubiquitin protease activity (Scheel *et al.*, 2003, Albrecht *et al.*, 2004, Chow *et al.*, 2004), and the UIM domains, which bind polyubiquitin proteins (Burnett *et al.*, 2003, Donaldson *et al.*, 2003), implicate Atx3 in ubiquitin-proteasome system (UPS). Atx3 is a transiently associated multiubiquitin chain recognition subunit in the proteasome that receives ubiquitinated substrates (Doss-Pepe *et al.*, 2003, Chai *et al.*, 2004). Moreover, Atx3 is a deubiquitinating enzyme (DUB), functioning as a polyubiquitin chain-editing enzyme that restores free and reusable ubiquitin (Ub) cellular levels (Berke *et al.*, 2005, Mao *et al.*, 2005). Thus, Atx3 is able to rescue proteins from degradation by eliminating the polyubiquitin chain, the substrate recognition signal for UPS degradation, or to stimulate protein break-down by removal of polyubiquitin upon substrate ligation to proteasome, allowing its degradation. Accordingly, mutation in Atx3 predictive catalytic sites or Atx3 knockout causes a marked increase of ubiquitinated proteins in cells (Berke *et al.*, 2005, Schmitt *et al.*, 2007). Additionally, Atx3 is suggested to be involved in transcriptional regulation, since it interacts with transcriptional co-activators and binds to DNA sequences, repressing transcription (Chai *et al.*, 2001, Evert *et al.*, 2006a). Moreover, Atx3 appears to be involved in the cellular response to heat stress (Araujo *et al.*, 2011, Rodrigues *et al.*, 2011) and in the cytoskeleton organization (Rodrigues *et al.*, 2010).

Taking into account these cellular functions, it has been speculated that the loss of normal Atx3 may contribute to MJD pathogenesis. Homozygous MJD patients have an earlier onset and a more severe phenotype than heterozygous patients (Carvalho *et al.*, 2008); the same is observed in transgenic mice of MJD (Boy *et al.*, 2010). However, mouse and *C. elegans* Atx3 knockouts show no impairments in viability, no morphologic abnormalities and no signs of neurodegeneration (Rodrigues *et al.*, 2007, Schmitt *et al.*, 2007), suggesting some redundancy in the functions of Atx3. Even though a study in a fly model of MJD showed that wild-type Atx3 was able to protect against the polyQ neurotoxicity (Warrick *et al.*, 2005), this neuroprotection was not observed in rodent models of the disease (Alves *et al.*, 2010, Hubener and Riess, 2010).

Therefore, it is more widely accepted that the polyQ expanded protein causes disease mainly by a dominant gain-of-function mechanism. In fact, mutant Atx3 intranuclear inclusions recruit several other proteins, like Ub (Paulson *et al.*, 1997b), proteasome subunits

(Chai *et al.*, 1999b), chaperones (Chai *et al.*, 1999a) and transcriptional co-activators (McCampbell *et al.*, 2000, Chai *et al.*, 2001), possibly affecting several cellular pathways having potentially toxic outcomes.

#### 1.1.1.1.6. Animal models of MJD

The identification of the disease-causing mutation allowed the development of animal models used to get further insight into the disease pathogenic mechanisms and to evaluate the potential of therapeutic strategies.

Invertebrate animals like *Drosophila melanogaster* (fruit fly) and *Caenorhabditis elegans* (worm) have been used to model MJD, since these models present the advantages of being simple, with reduced time requirements, ease of maintenance and genetic manipulation which make them interesting to large-scale screening studies. Warrick and colleagues created a MJD Drosophila model expressing full length or a truncated form of mutant Atx3. These flies showed locomotor dysfunction, formation of intranuclear aggregates and neuronal loss, resulting in premature death (Warrick *et al.*, 1998, Kim *et al.*, 2004, Warrick *et al.*, 2005). In worms, the expression of both full length and truncated forms of expanded Atx3 in the nervous system promoted the establishment of motor dysfunction, aggregation of the mutated protein and neuronal abnormalities (Khan *et al.*, 2006, Teixeira-Castro *et al.*, 2011).

Rodents are the most commonly used animals to model MJD (summarized in table 1.2), especially mice, since these small mammals share important molecular, anatomical and physiological similarities with humans.

**Table 1.2 – Rodent models of MJD**

| Animal | Transgene/promoter | Neuropathology | Phenotype | Ref. |
|---|---|---|---|---|
| Mouse | MJD1 fragment with 79 CAGs / L7 promoter – expression in Purkinje cells. | Atrophic cerebellum – neuronal cell loss and degeneration. | Ataxic postures and gait disturbance. | (Ikeda *et al.*, 1996) |
| | Full length MJD1 YAC with 76 or 84 CAGs / its own regulatory elements – ubiquitous expression. | Ubiquitinated NIIs in cerebellar neurons. Degeneration and mild gliosis of dentate and pontine nerve nuclei. | Mild and progressive: abnormal gait, tremor, hypoactivity, limb clasping, reduced grip strength. Slow weight gain. | (Cemal et al., 2002) |
| | Full length MJD1 with 71 CAGs / mouse prion protein promoter – expression throughout the brain. | NIIs in deep cerebellar and pontine nuclei and spinal cord. Abundant mutant atx3 fragments. | Progressive postural instability, gait and limb ataxia. Weight | (Goti *et al.*, 2004, Colomer Gould *et al.*, 2006) |

| | | | |
|---|---|---|---|
| | No detected neuronal loss. | loss. Premature death. | |
| Full length MJD1 with 70 or 148 CAGs, with or without NES and NLS / murine prion protein promoter – expression in several CNS regions. | Ubiquitinated NIIs in several brain regions. Shrinkage of Purkinje cells. | Severe and progressive: tremor, reduced motor and exploratory activity. Premature death. | (Bichelmeier *et al.*, 2007) |
| HA-tagged full-length MJD1 with 79 CAGs / mouse prion protein promoter – expression in several CNS regions. | NIIs in dentate and pontine nuclei and *substantia nigra*. Neuronal dysfunction in cerebellum. | Motor incoordination, hypoactivity, ataxic wide-based gait, abnormal hunchback posture, weight loss. | (Chou *et al.*, 2008) |
| HA-tagged truncated MJD1 with 69 CAGs / L7 promoter – expression in Purkinje cells and cerebellar nuclei. | Ubiquitinated inclusions. Cerebellar atrophy, disarrangement of Purkinje cells. | Severe ataxia. | (Torashima *et al.*, 2008, Oue *et al.*, 2009) |
| Full-length MJD1 with 77 CAGs / hamster prion protein promoter, using the Tet-Off system – expression in cerebellum, striatum, cortex, hippocampus, brainstem and pons. | NIIs in cerebellar cortex. Neuronal dysfunction in the cerebellum. | Reduced anxiety, hyperactivity, lower body weight gain, clasping, altered gait, impaired motor coordination. | (Boy *et al.*, 2009) |
| Full-length MJD1 gene with 148 CAGs / huntingtin promoter – ubiquitous expression in the brain. | NIIs in pons and cerebellum. Cell degeneration of Purkinje cells. | Hyperactivity, reduced motor coordination, impaired motor learning. | (Boy *et al.*, 2010) |
| Full-length MJD1 with 94 CAGs / cytomegalovirus (CMV) promoter – ubiquitous expression in CNS and periphery. | No large aggregates were found. Neurodegeneration in the absence of necrosis or apoptosis n thalamus, dentate and pontine nuclei. Astroglyosis. | Motor coordination impairment. | (Silva-Fernandes *et al.*, 2010) |
| Truncated N-terminal Atx3 cDNA (mutation in the mouse Atx3 gene) – expression in several brain regions. | Prominent extranuclear protein aggregates. Neuronal cell death. | Tremor, clasping, gait ataxia, weight loss. Premature death. | (Hubener *et al.*, 2011) |
| Myc-tagged full-length MJD1 with 72 CAGs / phosphoglicerate kinase 1 | Ubiquitinated NIIs. Loss of neuronal markers. | - | (Simões *et al.*, 2012) |

| | | | | |
|---|---|---|---|---|
| | (PGK) promoter encoded by lentiviral (LV) vectors injected in striatum. | Condensation of cell nucleus. | | |
| | Myc-tagged full-length MJD1 with 72 CAGs / PGK promoter encoded by LV vectors injected in cerebellum. | Ubiquitinated NIIs. Loss of neuronal markers. Shrinkage and degeneration of Purkinje cells. | Reduced motor coordination, wide-base ataxic gait, hyperactivity. | (Nobrega *et al.*, 2013) |
| | Full-length MJD1 with 135 CAGs / CMV promoter – ubiquitous expression in CNS and periphery. | Reduced brain weight. Neuronal loss in the pontine nuclei. | Abnormal gait and body posture, limb clasping, limb tonus deficit, lower body weight gain. | Silva-Fernandes *et al.*, 2014 |
| Rat | Myc-tagged full-length MJD1 with 72 CAGs / PGK promoter encoded by LV vectors injected in striatum or *substantia nigra*. | Ubiquitinated NIIs. Loss of neuronal markers. Condensation of cell nucleus. | - | (Alves *et al.*, 2008b) |

Some mouse and rat MJD models are listed along with their expressed transgene and the neuropathology and phenotype presented by transgenic animals.

This variety of transgenic rodent models due to the use of different Atx3 transcripts with a wide range of CAG repetitions and the use of different promoters allowing the expression of the transgene in several brain regions, promotes significant advances in the knowledge of this disorder. These models confirmed the importance of the number of CAG repeats in the disease severity: a longer CAG expansion caused a more aggressive phenotype and neurodegeneration (Bichelmeier *et al.*, 2007, Silva-Fernandes *et al.*, 2010, Silva-Fernandes *et al.*, 2014). Moreover, there is no clear correlation between NIIs formation and MJD-like symptoms. In fact, Boy and colleagues showed that transgenic mice have motor dysfunction before the formation of NIIs (Boy *et al.*, 2010), and Maciel's group reported that neurodegeneration occurred in the absence of NIIs (Silva-Fernandes *et al.*, 2010). Furthermore, it became evident that truncated forms of mutant ataxin-3 allowed a more severe pathology with an earlier age of onset of behavior abnormalities (3-4 weeks of age), whereas the expression of the full-length mutated protein promoted a later onset (some symptoms start at 2 months of age but others only manifest in 1-year-old mice) and a milder phenotype (Ikeda *et al.*, 1996, Cemal *et al.*, 2002, Goti *et al.*, 2004, Bichelmeier *et al.*, 2007, Chou *et al.*, 2008, Torashima *et al.*, 2008, Boy *et al.*, 2009, Boy *et al.*, 2010, Silva-Fernandes *et al.*, 2010, Silva-Fernandes *et al.*, 2014). Additionally, not only the C-terminal polyQ-containing fragment is the responsible for the establishment of MJD, since an N-terminal fragment of Atx3 was able promote neurodegeneration, causing behavioral abnormalities and culminating in premature death (Hubener *et al.*, 2011). Bichelmeier and colleagues

further explored the importance of the subcellular localization of the mutant Atx3, showing that its nuclear localization obtained by the expression of a NLS (nuclear localization signal) exacerbated the pathological phenotype compared to the mice that expressed a NES (nuclear export signal) (Bichelmeier *et al.*, 2007). Finally, using a MJD transgenic mouse with a Tet-Off system it was shown that it was possible to revert motor symptoms after shutting-off the expression of the mutated protein (Boy *et al.*, 2009).

MJD lentiviral (LV)-based animal models are able to overcome some limitations of transgenic mouse model, allowing genetic modeling of the disease in a faster, controlled and selective way (this topic will be further discussed in section 1.3).

Up to now, no model has completely recapitulated the MJD neuropathology and phenotype. Nevertheless, the currently available models have gave further insights and a better understanding of the disease pathogenesis.

### 1.1.2. Pathogenesis

#### 1.1.2.1. Neuronal loss, neurogenesis impairment and reduction of trophic factors levels

Ultimately, loss of neurons is the culprit of neurodegenerative diseases. In fact, neuronal replacement therapies have already been reported in an attempt to cope with neuronal death and alleviate the motor symptoms in HD (Bachoud-Levi *et al.*, 2006), PD (Dell'Anno *et al.*, 2014), SCA1 (Chintawar *et al.*, 2009) and MJD (Mendonca *et al.*, 2015). Moreover, it was already shown that after an ischemic injury induced by a stroke (Arvidsson *et al.*, 2002), brain traumatic injury (Urrea *et al.*, 2007) or an excitotoxic lesion caused by quinolinic acid injection (Gordon *et al.*, 2007) the adult brain can recruit endogenous neural proliferative cells to the damaged area, where they then express markers of developing and mature neurons.

Active generation of new neurons occurs throughout life, mostly in subventricular zone (SVZ) of the lateral ventricles and in subgranular zone (SGZ) of the dentate gyrus in the hippocampus (Ming and Song, 2005), and in a lower rate in other areas of the brain: hypothalamus (Kokoeva *et al.*, 2007, Cheng, 2013), neocortex (Gould *et al.*, 2001, Dayer *et al.*, 2005), striatum (Bedard *et al.*, 2006, Luzzati *et al.*, 2006), amygdala (Bernier *et al.*, 2002), *substantia nigra* (Zhao *et al.*, 2003) and cerebellum (Manohar *et al.*, 2012). Adult neurogenesis raises the possibility of restoring the diminished neuronal populations in neurodegenerative disorders. Neurogenesis can be an intrinsic compensatory mechanism to self-repair, though the effect of neurodegeneration on adult neurogenesis might be very

complex. Some studies have already pointed out alterations of adult neurogenesis in AD, PD and HD patients and mouse models, yet there are some conflicting reports (reviewed in (Winner and Winkler, 2015)). Despite methodological considerations that could be accounted for some contrasting results, it is reasonable to believe that these disorders stimulate neurogenesis as a self-repair mechanism, increasing the number of proliferating neural cells that then migrate to the damaged areas. However, regarding the continuous and progressive neuronal loss, it appears that the level of neurogenesis is insufficient to compensate for the progressive cell loss observed in the neurodegenerating brain. This could be simply due to a greater rate or extent of cell loss than the cell replacement capacity, but it is possible that these newborn neurons cannot survive or are not able to integrate the neuronal circuitry and be functional. In fact, the pathologic mechanisms of neurodegenerative diseases may create an aggressive environment characterized by the presence of inhibitory factors, such as inflammatory cues (Ekdahl *et al.*, 2003), and/or by inadequate levels of local trophic support necessary for neuronal differentiation and survival. Actually, brain-derived neurotrophic factor (BDNF), a neurotrophin that promotes neuronal survival and differentiation (reviewed in (Baydyuk, 2012)), is downregulated in the most affected brain areas of AD (Hock *et al.*, 2000), HD (Ferrer *et al.*, 2000) and SCA1 (Hourez *et al.*, 2011, Takahashi *et al.*, 2012). Additionally, TrkB (BDNF receptor) conditional knock-out mice develop ataxia (Johnson *et al.*, 2007). Furthermore, BDNF up-regulates the activity of survival genes and inhibits the function of the proteins that lead to programmed cell death (reviewed in (Baydyuk, 2012)). In fact, increasing BDNF levels improves motor phenotype and protects against cell atrophy and degeneration in HD (Xie *et al.*, 2010b) and SCA1 (Hourez *et al.*, 2011) transgenic mice.

#### 1.1.2.2. Quality control system failure

Protein misfolding and aggregation causes neurofibrillary tangles and amyloid β plaques in AD (McKhann *et al.*, 1984), α-synuclein aggregates that constitute Lewy bodies in PD (Jellinger, 1991) and expanded polyglutamine protein aggregates in PolyQ diseases (DiFiglia *et al.*, 1997, Skinner *et al.*, 1997, Holmberg *et al.*, 1998, Ishikawa *et al.*, 1999, Koyano *et al.*, 1999, McCampbell *et al.*, 2000, Nakamura *et al.*, 2001). This accumulation of abnormal proteins in neurons represents a continual burden on quality control systems in cells. In physiological conditions, cells count with machinery that allow misfolded or dysfunctional proteins clearance: ubiquitin-proteasome pathways and autophagy (reviewed in (Rubinsztein, 2006)).

For a protein to be degraded by the UPS it needs to be tagged by multiple Ub molecules (forming a polyubiquitin chain). This degradation signal is then recognized by the 26S proteasome complex, where the protein is proteolytically degraded to small peptides,

after a series of DUBs mediate the disassembly of the polyubiquitin chains, restoring the levels of free and reusable Ub (reviewed in (Ciechanover and Brundin, 2003)). The UPS is a very important pathway for cell homeostasis. It was shown that genetic depletion of the 26S proteasome or use of proteasome inhibitors in cells and mouse brain neurons induces extensive neurodegeneration, aggregates formation and motor deficits (Chai *et al.*, 1999b, Jana *et al.*, 2001, Wang *et al.*, 2007, Bedford *et al.*, 2008, Xie *et al.*, 2010a). The UPS has been implicated in neurodegenerative diseases. Indeed, inclusion bodies of AD, PD and polyQ diseases are ubiquitinated and contain proteasome components (Mori *et al.*, 1987, Perry *et al.*, 1987, Fergusson *et al.*, 1996, DiFiglia *et al.*, 1997, Paulson *et al.*, 1997b, Cummings *et al.*, 1998, Holmberg *et al.*, 1998, Chai *et al.*, 1999b, Koyano *et al.*, 1999, McNaught *et al.*, 2002, Schmidt *et al.*, 2002, McNaught *et al.*, 2003, Mandel *et al.*, 2005). These features may reflect failed attempts by the UPS to remove the abnormal protein. On the other hand, it can indicate that these damaged proteins could inhibit the UPS, hampering the degradation of other proteins. In fact, several studies show that the proteasome function is inhibited by the accumulation of amyloid β (Keller *et al.*, 2000, Keck *et al.*, 2003, Almeida *et al.*, 2006), mutant androgen receptor (Mandrusiak, 2003), mutant α-synuclein (McNaught and Jenner, 2001, Tanaka *et al.*, 2001), mutant huntingtin (Bence *et al.*, 2001, Holmberg *et al.*, 2004, Seo *et al.*, 2004, Bennett *et al.*, 2007, Wang *et al.*, 2008), mutant ataxin-1 (Park *et al.*, 2005) and mutant Atx3 (Tsai *et al.*, 2003). Furthermore, the stimulation of UPS activity enhances the rate of expanded polyQ-protein degradation, decreasing the formation of aggregates, and reduces cell degeneration, both *in vitro* and *in vivo* studies (Jana *et al.*, 2005, Al-Ramahi *et al.*, 2006, Adachi *et al.*, 2007).

The UPS is complemented by another quality control mechanism designated autophagy, which includes three different types: chaperone-mediated autophagy (CMA), microautophagy and macroautophagy. In macroautophagy (hereafter referred to only as autophagy), cytosolic components are sequestered into *de novo* formed double membrane vesicles, named autophagosome, that then fuses with lysosomes, generating autophagolysosomes, and their contents are then degraded by acidic lysosomal hydrolases (reviewed in (Wong and Cuervo, 2010)).

Autophagy is also very important in protein homeostasis. Loss of autophagy causes neurodegeneration even in the absence of any disease-associated mutated protein. In fact, it was shown that mice lacking either Atg5 or Atg7 (two proteins with key roles in autophagy) in the CNS develop progressive deficits in motor coordination, abnormal intracellular proteins accumulation and massive neuronal loss culminating in premature death (Hara *et al.*, 2006, Komatsu *et al.*, 2006). Alterations in autophagy have been shown to occur in neurodegenerative diseases. Using both postmortem diseased brain and animal models, it was observed lysosomal abnormalities (Cataldo *et al.*, 1994), autophagosome accumulation (Boland *et al.*, 2008, Spencer *et al.*, 2009, Nascimento-Ferreira *et al.*, 2011) and alterations

in levels of proteins involved in autophagic pathway (Shibata *et al.*, 2006, Pickford *et al.*, 2008, Vig *et al.*, 2009, Duncan *et al.*, 2010, Heng *et al.*, 2010, Jaeger *et al.*, 2010, Nascimento-Ferreira *et al.*, 2011), which could reflect a downstream block of autophagic flux, in AD, PD and polyQ disorders. Nevertheless, autophagy is able to prevent, at least at some extent, the aggregation and their induced toxicity in these disorders – the use of autophagy inhibitors or the autophagic genes silencing in disease models caused an increase in aggregates formation (Bi *et al.*, 1999, Lee *et al.*, 2004, Jia *et al.*, 2007, Montie *et al.*, 2009, Jaeger *et al.*, 2010, Watanabe *et al.*, 2012). On the other hand, stimulation of autophagy reduced the cellular accumulation of amyloid β (Pickford *et al.*, 2008, Jaeger *et al.*, 2010, Spilman *et al.*, 2010, Majumder *et al.*, 2011), mutant α-synuclein (Pan *et al.*, 2008, Spencer *et al.*, 2009), mutant huntingtin (Ravikumar *et al.*, 2004, Shibata *et al.*, 2006), mutant ataxin-1 (Berger *et al.*, 2006), mutant Atx3 (Menzies *et al.*, 2010, Nascimento-Ferreira *et al.*, 2011, Nascimento-Ferreira *et al.*, 2013) and mutant androgen receptor (Montie *et al.*, 2009), presenting a neuroprotective effect against the disease-like symptoms and neurodegeneration.

#### 1.1.2.3. Excitotoxicity and deregulation of calcium homeostasis

The concept of excitotoxicity was first introduced in 1978, describing it as a process of neuronal death caused by excessive or prolonged activation of glutamate receptors: NMDA receptors, AMPA/Kainate receptors and metabotropic receptors (Olney, 1978, Lau and Tymianski, 2010). Prolonged activation of these receptors leads to continuous cationic fluxes into the postsynaptic neurons, causing persistent depolarization of the plasmatic membrane, which ultimately translates into neuronal $Ca^{2+}$ overload. Physiologically, tight homeostatic mechanisms regulate intracellular $Ca^{2+}$ concentrations, like calcium sequestration by mitochondria, preventing pathological rises of $Ca^{2+}$ in the cytosol. Excessive $Ca^{2+}$ influx results in mitochondrial failure, which in turn leads to energy deprivation and reactive oxygen species (ROS) overproduction causing oxidative stress. Their cytotoxic effects join the activation of calcium-sensitive enzymes such as proteases, endonucleases and phospholipases, triggering signaling cascades that promote neuronal death, either by apoptosis or necrosis (reviewed in (Dong *et al.*, 2009)).

Excitotoxicity and alterations in $Ca^{2+}$ homeostasis have been implicated in neurodegenerative diseases. Several studies showed that amyloid β induces excitotoxicity in cortical and hippocampal neuronal cultures by, at least in part, inhibiting the uptake of glutamate (Mattson *et al.*, 1992, Brorson *et al.*, 1995, Gray and Patel, 1995, Harkany *et al.*, 2000). Moreover, declines in endogenous antioxidants were observed in AD patients' brains, rendering neurons more vulnerable to excitotoxic insults (Ansari and Scheff, 2010).

Furthermore, antioxidants and NMDA receptor antagonists showed neuroprotection against amyloid β toxic effects (Behl *et al.*, 1994, Miguel-Hidalgo *et al.*, 2002). Additionally, glutamate receptor antagonists improved motor impairments of PD patients (Rabey *et al.*, 1992, Uitti *et al.*, 1996, Del Dotto *et al.*, 2001). In this case, excitotoxicity could possibly be explained by an increased cortical glutamatergic input to the striatum due to the striatal dopamine depletion that, this way, was not able to depress the release of glutamate from cortical afferents (Calabresi *et al.*, 1993, Bamford *et al.*, 2004, Vaarmann *et al.*, 2013). Indeed, lesions of the motor cortex decreased the striatal glutamate levels, thus reversing the degeneration of striatal neurons (Garcia *et al.*, 2010). Alterations in calcium homeostasis are also present in polyQ disorders, such as HD (Levine *et al.*, 1999, Sun *et al.*, 2001, Zeron *et al.*, 2004, Tang *et al.*, 2005), SCA1 (Lin *et al.*, 2000, Serra *et al.*, 2004), SCA6 (Matsuyama *et al.*, 2004) and MJD (Chen *et al.*, 2008, Koch *et al.*, 2011, Goncalves *et al.*, 2013, Konno *et al.*, 2014), revealing an increase in the intracellular $Ca^{2+}$ levels. Moreover, mitochondrial dysfunction has been pointed as a cause of excitotoxicity (Beauchemin *et al.*, 2001, Panov *et al.*, 2002, Tsai *et al.*, 2004, Chou *et al.*, 2006, Oliveira *et al.*, 2006, Shehadeh *et al.*, 2006, Wang *et al.*, 2006, Fernandes *et al.*, 2007, Zhang *et al.*, 2008, Ranganathan *et al.*, 2009, Yu *et al.*, 2009, Laco *et al.*, 2012, Kazachkova *et al.*, 2013), leading also to an increase of ROS production (Browne *et al.*, 1997, Kim *et al.*, 2003, Puranam *et al.*, 2006, Ranganathan *et al.*, 2009, Yu *et al.*, 2009, Araujo *et al.*, 2011). Furthermore, it was shown that NMDA receptors antagonists reduced motor deficits of HD (Lucetti *et al.*, 2002, Beister *et al.*, 2004) and that MJD transgenic mice treated with a stabilizer of intracellular calcium levels have an improved motor performance and a reduced neuronal loss (Chen *et al.*, 2008).

#### 1.1.2.4. Neuroinflammation

Microglia are considered the resident immune cell population of the CNS (Garden and Moller, 2006). Upon CNS injury, microglia cells become activated and their morphology shifts into ameboid-shapped cells which present and up-regulated catalogue of surface molecules (reviewed in (Block *et al.*, 2007)). One of the earliest response of microglia is migration to the site of neural injury, where they proliferate and secrete pro-inflammatory cytokines, such as interleukin-1 beta (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNFα), which help to drive the resolution of the injury. These molecules also signal to recruit astrocytes, thus amplifying the immune response. The activated microglia also release free radicals, nitric oxide and metalloproteases necessary for tissue repair (Schultzberg *et al.*, 2007, Wee Yong, 2010). Physiologically, when the activating stimulus disappears, microglia participates in the down-modulation of the inflammation releasing anti-inflammatory cytokines and trophic factors, important to the recovery process: anti-inflammatory cytokines

signal for microglia apoptosis, and some remaining microglial cells return to their resting morphology (Garden and Moller, 2006, Czlonkowska and Kurkowska-Jastrzebska, 2011). However, under abnormal circumstances, when the stimulus continues to be present or when the immune response becomes deregulated, chronic inflammation can take place, microglia become overactivated and the pro-inflammatory factors prove to be harmful for the CNS cells (Block *et al.*, 2007).

PET imaging studies showed increased levels of activated microglia in the temporoparietal cortex of AD patients (Cagnin *et al.*, 2001, Vehmas *et al.*, 2003) and also astrocytic activation, that in a late stage is associated with the dementia degree (Vehmas *et al.*, 2003). PET imaging also revealed higher accumulation of activated microglia in the basal ganglia and *substantia nigra* in PD patients (Ouchi *et al.*, 2005, Gerhard *et al.*, 2006) and in a 6-hydroxydopamine (6-OHDA) lesion rat model (Cicchetti *et al.*, 2002), although it is not consensual the correlation with the disease severity. Moreover, significant increases in activated microglia in the striatum and cerebral cortex in HD patients (Pavese *et al.*, 2006) and presymptomatic gene carriers (Tai *et al.*, 2007) were also revealed by PET scans, being the amount of activated microglia associated with the severity of clinical disease. An increase of reactive astrocytes and activated microglia was also observed in pons and globus pallidus of MJD patients (Evert *et al.*, 2001, Horimoto *et al.*, 2011) and in striatum and *substantia nigra* of MJD transgenic mouse models (Silva-Fernandes *et al.*, 2010, Goncalves *et al.*, 2013). Moreover, serum levels of GFAP were observed to be higher in MJD patients (Shi *et al.*, 2015). Likewise, a study in several mouse models revealed that astrocytes and microglia were also activated early in SCA1 pathogenesis, even in the absence of neuronal death (Cvetanovic *et al.*, 2015). Besides, increased levels of pro-inflammatory cytokines were found in CSF of AD patients (Blum-Degen *et al.*, 1995), in cerebral cortex and hippocampus of an AD mouse model (Tehranian *et al.*, 2001), in the CSF of PD patients (Blum-Degen *et al.*, 1995), and striatum of a PD mouse model (Ciesielska *et al.*, 2003), in HD patients' plasma and in an HD mouse model serum (Dalrymple *et al.*, 2007, Bjorkqvist *et al.*, 2008) and in pons and cerebellum of MJD patients (Evert *et al.*, 2001, Evert *et al.*, 2006b).

Therefore, there are several studies showing that the major neurodegenerative disorders have an inflammatory component. Experiments using cortical neuronal and glial cultures suggest that neuroinflammation may be a precipitator of neurodegeneration (Del Bo *et al.*, 1995, Qin *et al.*, 2002). Despite that, neuronal loss can in itself result in activation of microglia, initiating a self-propagating sequence of cell damage. Thus, therapeutic strategies that suppress inflammation should be considered to prevent the exacerbation of these neurodegenerative disorders. Epidemiological studies show that populations that take anti-inflammatory drugs on a sustained basis have a reduced risk of developing AD (reviewed in (McGeer and McGeer, 2007)). Moreover, the use of drugs with anti-inflammatory effects decrease the neuronal damage and improve the phenotype of mice that model AD (McGeer

and McGeer, 2007) and PD (Kurkowska-Jastrzebska *et al.*, 2002, Kurkowska-Jastrzebska *et al.*, 2004); the results in an HD mouse model are contradictory (Chen *et al.*, 2000, Smith *et al.*, 2003).

### 1.2. Neuropeptide Y (NPY) and NPY receptors

Neuropeptide Y (NPY), a 36 amino acid peptide with several tyrosines first isolated from porcine brain more than three decades ago (Tatemoto, 1982, Tatemoto *et al.*, 1982), is expressed throughout the body, including the brain. Due to their homology NPY was grouped with pancreatic polypeptide (PP) and peptide YY (PYY), 2 gut hormones, in the same family (Michel *et al.*, 1998). NPY is highly conserved among mammals throughout the evolution (Larhammar, 1996).

The NPY human gene is located in chromosome 7 at the locus 7p15.1 (Cerda-Reverter and Larhammar, 2000). Pre-pro-NPY, a 97 amino acid peptide generated after translation, is directed to the endoplasmic reticulum (ER), where it is proteolytically processed and a 27 amino acid fragment is removed resulting in the production of Pro-NPY. This NPY precursor is formed by $NPY_{1-39}$ and C-flanking peptide of NPY (CPON); these two Pro-NPY components are dissociated through a cleavage promoted by prohormone convertases. $NPY_{1-39}$ is further processed by a carboxypeptidase B (CPB) generating $NPY_{1-37}$, which is a substrate for the enzyme peptidylglycine alpha-amidating monooxygenase (PAM), leading to the formation of the biologically active C-terminally amidated $NPY_{1-36}$ (normally designated only by NPY) (Medeiros Mdos and Turner, 1996).

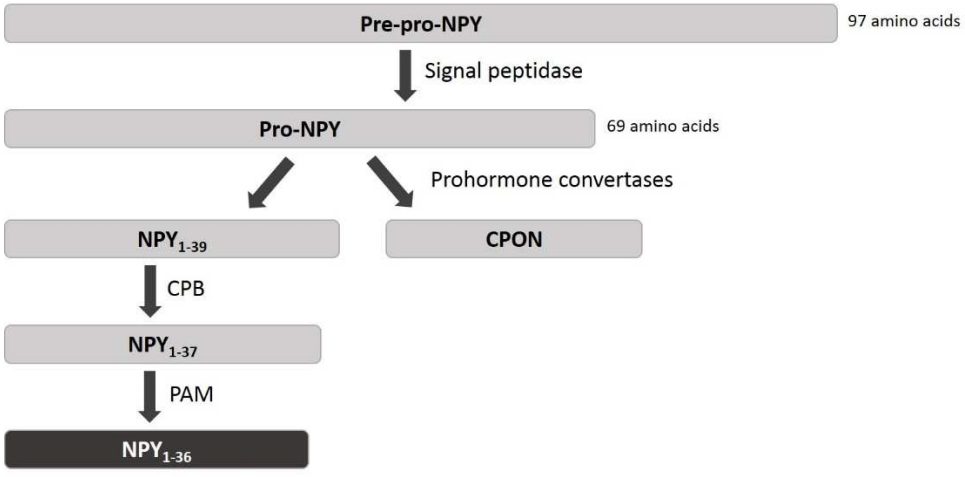


**Figure 1.4 – Scheme of the biosynthesis of NPY.** CPON: C-flanking peptide of NPY; CPB: carboxypeptidase B; PAM: peptidylglycine alpha-amidating monooxygenase.

The mature NPY can be ultimately cleaved mainly by the dipeptidyl peptidase IV (DPP-IV) and, in a lesser extent, by the aminopeptidase P (AmP), resulting in $NPY_{3-36}$ and $NPY_{2-36}$ fragments, respectively (Medeiros and Turner, 1994, Medeiros Mdos and Turner, 1996). Both fragments can also be degraded by endopeptidase neutral-24-11, originating biologically inactive peptides $NPY_{1-20}$ and $NPY_{31-36}$ (Yaron and Mlynar, 1968, Medeiros and Turner, 1994, Medeiros Mdos and Turner, 1996).

NPY is abundantly and unequally distributed in several brain regions (Table 1.3). NPY levels were evaluated by radioimmunoassay (RIA) and NPY-ir was assessed by immunohistochemistry (IHC) (Adrian *et al.*, 1983, Allen *et al.*, 1983, de Quidt and Emson, 1986). The results show that amygdala, hippocampus, hypothalamus and striatum contain the higher levels of NPY. This NPY protein distribution correspond to the distribution of NPY mRNA (Caberlotto *et al.*, 2000, Allen Institute for Brain Science, 2014).

**Table 1.3 – NPY distribution and content in human and rodents brain.**

| CNS region | Human | Rodents |
|---|---|---|
| Amygdala | +++ | ++++ |
| Cerebellum | + | + |
| Cerebral cortex | + | +++ |
| *Globus pallidus* | + | ++ |
| Hippocampus | ++ | +++ |
| Hypothalamus | ++ | ++++ |
| Striatum | ++++ | +++ |
| *Substantia nigra* | + | + |
| Thalamus | + | + |
| Ref. | (Adrian *et al.*, 1983, Caberlotto *et al.*, 2000) | (Allen *et al.*, 1983, de Quidt and Emson, 1986, Allen Institute for Brain Science, 2014) |

These data does not compare NPY levels between human and rodents brains. ++++, very high levels; +++, high levels; ++, moderate levels; +, low levels.

As a neurotransmitter or a neuromodulator, NPY activates different NPY receptors in several brain regions and has different functions in the CNS.

NPY receptors are G-protein-coupled receptors, five of which have already been cloned from mammals: $Y_1$, $Y_2$, $Y_4$, $Y_5$ and y6 receptors (reviewed in (Michel *et al.*, 1998, Silva *et al.*, 2005)). These receptors are grouped into the same family due to the fact that they all bind to NPY, despite generally low sequence similarity; nevertheless, as NPY itself, they are very conserved across species (Larhammar *et al.*, 2001).

The $Y_1$ receptor, a 384 amino acid peptide, was the first cloned NPY receptor. Its human gene is located in chromosome 4q31 (Gehlert, 2004). Only the NPY N-terminal is essential for the activation of $Y_1$ receptor since the fragments $NPY_{2-36}$, $NPY_{3-36}$ and $NPY_{13-36}$,

which are cleaved in this terminal, present a low affinity to this receptor (Medeiros and Turner, 1994, Medeiros Mdos and Turner, 1996). The $Y_2$ receptor human gene is also located in chromosome 4. This peptide with 381 amino acids has high affinity to NPY and PYY as well as to NPY analogs cleaved in N-terminal, like $NPY_{2-36}$, $NPY_{3-36}$ and $NPY_{13-36}$ (Michel *et al.*, 1998). The $Y_4$ receptor, whose human gene is in chromosome 10, has higher affinity to PP than to PYY, and even smaller to NPY (Lundell *et al.*, 1995). The gene for $Y_5$ receptor is located in human chromosome 4 and overlaps with $Y_1$ receptor gene but is transcribed in the opposite direction (Gehlert, 2004). The $Y_5$ receptor is activated by NPY, NPY fragments NPY2-36 and NPY3-36 and PYY (Gerald *et al.*, 1996, Borowsky *et al.*, 1998, Michel *et al.*, 1998). The y6 receptor is present in some mammals, like mouse, rabbit, dog, cow and primates, including humans, but it is absent in rats (Burkhoff *et al.*, 1998); however, the y6 receptor is only functional in few species, excluding human (Matsumoto *et al.*, 1996, Dumont *et al.*, 1998, Michel *et al.*, 1998, Starback *et al.*, 2000), and its physiological functions are not known yet.

All NPY receptors have been described as post-synaptic, being the $Y_2$ receptor the only that has a pre-synaptic localization, where it may act as an autoreceptor (Colmers *et al.*, 1991, Stanic *et al.*, 2011). Each NPY receptor is present in different brain regions, although their levels may vary depending on the region (Caberlotto *et al.*, 1997, Gustafson *et al.*, 1997, Borowsky *et al.*, 1998, Caberlotto *et al.*, 1998, Naveilhan *et al.*, 1998, Nichol *et al.*, 1999, Neveu *et al.*, 2002, Wolak *et al.*, 2003, Stanic *et al.*, 2006, Oberto *et al.*, 2007). The $Y_4$ receptor is the least expressed of all four NPY receptors in the CNS (Kask *et al.*, 2002, Silva *et al.*, 2005).

The potential physiological roles of NPY receptors were already described by studies in rodent models deficient in various receptor subtypes (reviewed in (Lin *et al.*, 2004)) and by using the classical pharmacological tools (selective agonists and antagonists of NPY receptors). Moreover, some studies suggested the possible existence of NPY receptors homodimers and heterodimers (Dinger *et al.*, 2003, Silva *et al.*, 2005, Gehlert *et al.*, 2007). Besides, it is possible that additional mammalian NPY binding sites remain to be discovered.

NPY is contained mainly in GABA neurons (Aoki and Pickel, 1989, McDonald and Pearson, 1989). Some of these neurons contain also other neurotransmitters or neuromodulators, such as somatostatin, in striatum (Smith and Parent, 1986, Kowall *et al.*, 1987, Schwartzberg *et al.*, 1990, Dawson *et al.*, 1991, Figueredo-Cardenas *et al.*, 1996), cerebral cortex (Hendry *et al.*, 1984) and amygdala (McDonald, 1989, Schwartzberg *et al.*, 1990, McDonald *et al.*, 1995), and nitric oxide, in striatum (Dawson *et al.*, 1991, Figueredo-Cardenas *et al.*, 1996). This suggests that NPY's physiological roles in the CNS are very complex since it interacts with other effector systems.

### 1.2.1. NPY system in neurodegenerative disorders

*Alzheimer's disease*

NPY levels in AD patients were evaluated in several studies (summarized in Table 1.5). In postmortem human brain it has been shown by IHC that the morphology of cortical NPY-positive neurons was severely altered by the disease (Chan-Palay *et al.*, 1985), and their number was significantly decreased in both cerebral cortex and hippocampus (Beal *et al.*, 1986b, Chan-Palay *et al.*, 1986, Davies *et al.*, 1990). Moreover, autoradiographic studies showed that NPY receptors density in those brain regions was also decreased (Martel *et al.*, 1990). CSF of AD patients was also analyzed, by RIA, but the results concerning NPY concentrations were not consistent (Atack *et al.*, 1988, Sunderland *et al.*, 1991, Martignoni *et al.*, 1992, Edvinsson *et al.*, 1993, Heilig *et al.*, 1995). The discrepant findings could be explained by the type of NPY assay, since intra or extracellular processing of NPY can result in fragments that may or may not be detected by the assay (Nilsson *et al.*, 2001). The same explanation can be applied to the inconsistent results that were found in the peripheral plasma analysis (Koide *et al.*, 1995, Proto *et al.*, 2006). Besides, even though the pursuit of NPY as a peripheral biomarker of the disease is relevant, the role and origin of plasma NPY and its relation with central NPY remains to be clarified.

**Table 1.5 – NPY levels in AD.**

| Biological sample | NPY levels | Ref. |
|---|---|---|
| Human brain | Severely altered morphology of NPY-positive neurons in cerebral cortex and hippocampus. | (Chan-Palay *et al.*, 1985) |
| | ↓ NPY-ir in cerebral cortex and hippocampus. | (Beal *et al.*, 1986b, Chan-Palay *et al.*, 1986, Davies *et al.*, 1990) |
| | ↓ density of NPY binding sites in cerebral cortex and hippocampus. | (Martel *et al.*, 1990) |
| Human CSF | No changes in NPY concentration. | (Atack *et al.*, 1988, Sunderland *et al.*, 1991, Heilig *et al.*, 1995) |
| | ↓ NPY levels. | (Martignoni *et al.*, 1992, Edvinsson *et al.*, 1993) |
| Human plasma | ↓ NPY concentration. | (Koide *et al.*, 1995) |
| | Unchanged NPY concentration. | (Proto *et al.*, 2006) |
| Mouse models | PDAPP model: ↑ NPY levels in cerebral cortex and hippocampus | (Diez *et al.*, 2000) |
| | APP23 model: ↑ NPY levels in cerebral cortex and hippocampus. | (Diez *et al.*, 2003) |
| | PS1xAPP model: ↓ NPY neurons in hippocampus at early stage of disease | (Ramos *et al.*, 2006) |

Some studies in AD mouse models do not mimic the ones in human brain (Diez *et al.*, 2000, Diez *et al.*, 2003), possibly because transgenic models reproduce some but not all aspects of the disease – transgenic mice display extensive amyloid plaques and cognitive deficiencies but not the massive neuronal loss characteristic of the human disease. No studies were done in an attempt to evaluate de NPY receptors levels in these models.

It is likely that the loss of NPY in hippocampus and cerebral cortex of AD patients results from neurodegeneration occurring in these brain regions along with the progression of the disease.

*Parkinson's disease*

Studies that evaluated NPY levels in PD patients are summarized in Table 1.6. Allen *et al.* (1985) found no differences in NPY levels in hippocampus nor in cerebral cortex but Beal *et al.* (1988a) showed a 10-30% decrease in some cortical regions of PD patients, compared to controls. Regarding the striatum, by ISH analysis, NPY mRNA levels were higher in postmortem human brains of PD patients (Cannizzaro *et al.*, 2003). CSF NPY levels were also investigated, but the results were not consistent (Yaksh *et al.*, 1990, Martignoni *et al.*, 1992), probably because of, as discussed previously, the questionable reliability of NPY assay on CSF samples.

Rodent models used to study PD are based on the chemical destruction of the nigrostriatal dopaminergic neurons. PD rodent models obtained by striatal injection of 6-OHDA show an increased number of NPY-positive interneurons in the lesioned striatal hemisphere as compared to the intact one and to striata of control animals (Kerkerian *et al.*, 1986). Moreover, these changes were reversed by treatment with apomorphine, a dopaminergic agonist (Kerkerian *et al.*, 1988). The PD mouse model obtained by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) administration also presented a significant increase in NPY-ir, which was also reversed by L-DOPA treatment (Obuchowicz *et al.*, 2003).

**Table 1.6 – NPY levels in PD.**

| Biological sample | NPY levels | Ref. |
|---|---|---|
| Human brain | No changes in NPY levels in cerebral cortex and hippocampus. | (Allen *et al.*, 1985) |
| | ↓ NPY levels in some cortical regions. | (Beal *et al.*, 1988) |
| | ↑ NPY mRNA levels in striatum. | (Cannizzaro *et al.*, 2003) |
| Human CSF | No changes in NPY concentration. | (Yaksh *et al.*, 1990) |
| | ↓ NPY levels. | (Martignoni *et al.*, 1992) |
| Rodent models | ↑ NPY-positive interneurons in 6-OHDA lesioned striata. | (Kerkerian *et al.*, 1986, Kerkerian *et al.*, 1988) |
| | NPY-ir in the striatum of MPTP treated mice. | (Obuchowicz *et al.*, 2003) |

Despite some conflicting reports, the results suggest that the decreased tonic suppression of striatal NPY-positive interneurons by dopamine is responsible for NPY increased levels in PD. There is no information regarding the NPY receptors levels in PD patients or animal models.

*Huntington's disease*

Some studies have been performed to assess NPY brain levels in HD patients and rodent models (summarized in Table 1.7). Postmortem human brains were analyzed by RIA and IHC, and the authors observed an increase of NPY levels and a significantly higher number of NPY-positive neurons in HD striatum relative to controls (Dawbarn *et al.*, 1985, Beal *et al.*, 1988b). This NPY levels increase was also observed in HD frontal and temporal cortical regions (Beal *et al.*, 1988b, Mazurek *et al.*, 1997).

The HD rat model obtained by striatal lesion induced by a NMDA-receptor-specific excitotoxin, quinolinic acid (QA), exhibited less NPY-positive interneurons in the core of the lesion, but the cells in the transition state, in the periphery, were spared, as showed by IHC techniques (Boegman *et al.*, 1987). However, with RIA analysis, other authors did not observe changes in striatal NPY levels (Beal *et al.*, 1986a), which suggests that the spared NPY interneurons can be in an hyperactive state to compensate for the neuronal loss. QA was also used in rat striatal cultures to develop an *in vitro* model, and it was observed that NPY neurons were selectively spared (Kumar, 2004). No studies were conducted in an attempt to evaluate the NPY receptors levels in HD.

**Table 1.7 – NPY levels in HD.**

| Biological sample | NPY levels | Ref. |
|---|---|---|
| Human brain | ↑ NPY levels in striatum. | (Dawbarn *et al.*, 1985, Beal *et al.*, 1988b) |
| | ↑ number of NPY interneurons in striatum. | (Dawbarn *et al.*, 1985) |
| | ↑ NPY levels in frontal and temporal cortex. | (Beal *et al.*, 1988b, Mazurek *et al.*, 1997) |
| Rat models | Striatal QA lesions: spared NPY interneurons in transition state of the lesion and unchanged NPY levels. | (Beal *et al.*, 1986a, Boegman *et al.*, 1987) |
| *In vitro* models | Striatal cultures: spared NPY neurons. | (Kumar, 2004) |

The above mentioned findings are consistent with a sparing of NPY interneurons in HD, although the mechanism responsible for it is not yet known.

### 1.2.2. NPY functions and related mechanisms relevant in neurodegenerative disorders

### 1.2.2.1. NPY stimulates neurogenesis and increases trophic support

Various physiological stimuli dynamically regulate adult neurogenesis, including the proliferation of neural cells or progenitors, their migration, differentiation and fate determination, and the survival, maturation and integration of newborn neurons. These modulator factors are growth factors, neurotrophins, cytokines, hormones, adhesion molecules, transcription factors, neurotransmitters and neuropeptides (Zhao *et al.*, 2008), such as NPY. Different *in vitro* studies using primary cell cultures have showed that NPY has neuroproliferative potential in the olfactory epithelium (Hansel *et al.*, 2001), in the hippocampus (Howell *et al.*, 2003, Corvino *et al.*, 2014), in the SVZ (Agasse *et al.*, 2008) and in the retina (Alvaro *et al.*, 2008a), since exogenous NPY was able to increase the total number of neurons, paralleling the increase of BrdU incorporation rates. In these studies, using NPY receptors' agonists and antagonists, it was reported that this proliferative action is mediated preferentially by $Y_1$ receptor activation, although $Y_2$ receptor (in the SVZ and the retina) and $Y_5$ receptor (in the retina) can also have a role (Figure 1.5). Decressac *et al.* injected intracerebroventricularly (ICV) NPY in adult mice and confirmed the NPY proliferative effect, first in the SVZ (2009) and then in the SGZ (2011), mediated by $Y_1$ receptor activation. The authors also showed that NPY promotes the migration of the progenitor cells from SVZ to the olfactory bulb and to the striatum, where they can differentiate. Furthermore, ICV administration of NPY to a rat model of hippocampal neurodegeneration increases the number of new neurons that later functionally integrate into the local hippocampal circuits (Corvino *et al.*, 2014).

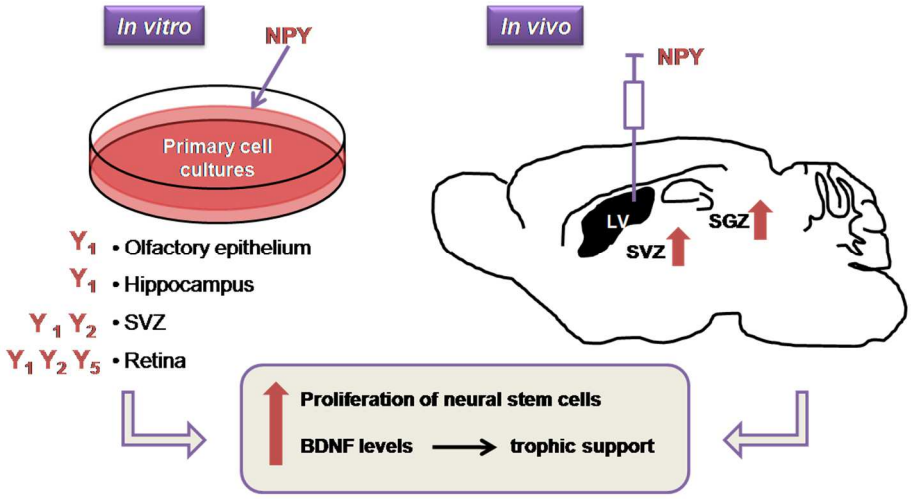


**Figure 1.5 – NPY stimulates neurogenesis and increases trophic support.** Several *in vitro* studies reveal that NPY stimulates proliferation of neural stem cells in olfactory epithelium, hippocampus, SVZ and retina, through the activation of primarily $Y_1$ receptor, but also $Y_2$ (in the SVZ and in the retina) and $Y_5$ (in the retina) receptors. ICV injection of NPY stimulates proliferation

of neural cells from SVZ and from SGZ, and its migration to olfactory bulb and to striatum where they differentiate. NPY overexpression leads to an up-regulation of BDNF levels, providing trophic support for these newborn cells.

A clear interaction between BDNF and NPY has already been described. Both *in vitro* and *in vivo* studies documented that BDNF overexpression robustly increases NPY mRNA and protein levels (Nawa *et al.*, 1993, Reibel *et al.*, 2000, Barnea and Roberts, 2001, Wirth *et al.*, 2005). The opposite correlation also occurs: NPY overexpression leads to an up-regulation of BDNF levels (Croce *et al.*, 2013). In fact, it was already shown that in rat primary cortical neurons exposed to amyloid β, an *in vitro* model of AD, NPY decreases neuronal cell death induced by amyloid β, restoring the nerve growth factor (NGF, a neurotrophin that supports survival and function of neuronal population) release by those neurons (Croce *et al.*, 2012) and also inducing an up-regulation of BDNF (Croce *et al.*, 2013). Moreover, the ICV injection of NPY in an HD mouse model enhanced the number of newborn neuroblasts in the SVZ, which improved the motor phenotype and increased the survival of these transgenic mice, leading the authors to suggest that NPY may have modulated the striatal BDNF expression (Decressac *et al.*, 2010).

Altogether, these results suggest that NPY administration can be a therapeutic strategy to compensate the cell loss of these neurodegenerative diseases, either by stimulating proliferation of progenitor cells and/or by promoting the production of adequate trophic support for the newly generated neurons.

#### 1.2.2.2. NPY stimulates autophagy

NPY is able to stimulate autophagy in mouse hypothalamic neuronal cell line and in rat differentiated hypothalamic neural cells, through $Y_1$ and $Y_5$ receptors activation, as shown by the analysis of levels of proteins involved in autophagic flux and the increase in the number of autophagosomes and autolysosomes. This autophagy stimulation was further confirmed *in vivo* by ICV administration of adeno-associated viral (AAV) vectors encoding NPY in arcuate nucleus of mice hypothalamus (Aveleira *et al.*, 2015). Interestingly, the authors also observed that in hypothalamic neurons NPY receptors antagonists inhibit autophagy stimulation induced by caloric restriction, suggesting that NPY receptors activation is one possible mechanism by which caloric restriction increases autophagy (Aveleira *et al.*, 2015). Since several studies show that autophagy is one of the main underlying mechanisms of caloric restriction beneficial effects, delaying neurodegenerative disease progression (Hansen *et al.*, 2005, Blagosklonny, 2010, Duan and Ross, 2010), NPY may mimic these neuroprotective effects mediated by autophagy stimulation (Minor *et al.*, 2009).

Although there is a lack of studies in neurodegenerative disorders models to investigate the potential of NPY to delay neurodegeneration through autophagy stimulation, the evidence that NPY is able to induce autophagy suggests that it may be used as a strategy to clear abnormal, misfolded proteins that cause AD, PD and polyQ diseases.

#### 1.2.2.3. NPY decreases excitotoxicity and regulates calcium homeostasis

Some studies suggest that NPY is a neuroprotective agent against excitotoxicity. Actually, it was already clearly shown the anticonvulsant properties of NPY in epileptic seizures caused by neuronal hyperexcitability (reviewed in (Noe *et al.*, 2012)). Studies reported an increase of endogenous NPY levels in hippocampus of patients with temporal lobe epilepsy (Furtinger *et al.*, 2001) and of rodent models of epilepsy (Schwarzer *et al.*, 1996, Vezzani *et al.*, 1996). Furthermore, NPY deficient-mice present prolonged seizure activity induced by excess excitation that was not controlled in a physiological manner, culminating in the death of these animals, unless NPY is ICV infused to stop the seizure (Baraban *et al.*, 1997). These lines of evidence strongly suggest that NPY is an endogenous compensatory response to overcome epileptic activity. In fact, NPY overexpression mediates powerful anticonvulsant effects in rats injected with kainic acid (Noe *et al.*, 2010). Accordingly, using hippocampal slices it was shown that NPY inhibits voltage-dependent $Ca^{2+}$ channels reducing intracellular calcium concentrations and thereby inhibiting synaptic transmission (Qian *et al.*, 1997). Moreover, studies in cell cultures revealed that NPY reduces the excitotoxic effects of glutamate and of AMPA and kainate receptors hyperactivation rescuing hippocampal, cortical and retinal cells from necrosis or apoptosis, through activation of $Y_2$ and $Y_5$ receptors (Qian *et al.*, 1997, Silva *et al.*, 2003, Wu and Li, 2005, Smialowska *et al.*, 2009, Santos-Carvalho *et al.*, 2013). These neuroprotective effects of NPY, against kainate-induced toxicity were further demonstrated in rodent models, reducing cell death in both hippocampus and retina (Smialowska *et al.*, 2009, Santos-Carvalho *et al.*, 2013). Moreover, NPY and particularly $Y_2$ and $Y_5$ receptors agonists can also attenuate the neurotoxic effects of drugs of abuse such as 3,4-methylenedioxymethamphetamine (MDMA), trimethyltin (TMT) and methamphetamine (METH), by blocking the glutamate release (Baptista *et al.*, 2012), modulating anti-apoptotic pathways as evidenced by an up-regulation of anti-apoptotic genes that translates into a decrease in the number of apoptotic cells (Thiriet *et al.*, 2005, Alvaro *et al.*, 2008b, Corvino *et al.*, 2012, Goncalves *et al.*, 2012) and by decreasing ROS production attenuating oxidative stress (Yarosh and Angulo, 2012), both in hippocampal or retinal cells cultures and in rodents (Figure 1.6).

In 2012, Decressac *et al*. demonstrated the neuroprotective effect of NPY or $Y_2$ receptor agonists in *in vitro* and *in vivo* models of PD – NPY increased the cell viability of

cultured neurons treated with 6-OHDA, and preserved the nigrostriatal dopaminergic pathway of a PD mouse model from degeneration, as evidenced by a preservation of rotational behavior of animals and of neuronal markers. The authors suggested that NPY exerts its protective effects at least in part by acting on glutamatergic cortical afferents to reduce excitotoxicity (Decressac *et al.*, 2012). Furthermore, in an *in vitro* model of HD, NPY treatment had a protective effect against mutant huntingtin-induced cell death, through the activation of the $Y_2$ receptor (Kloster *et al.*, 2014).

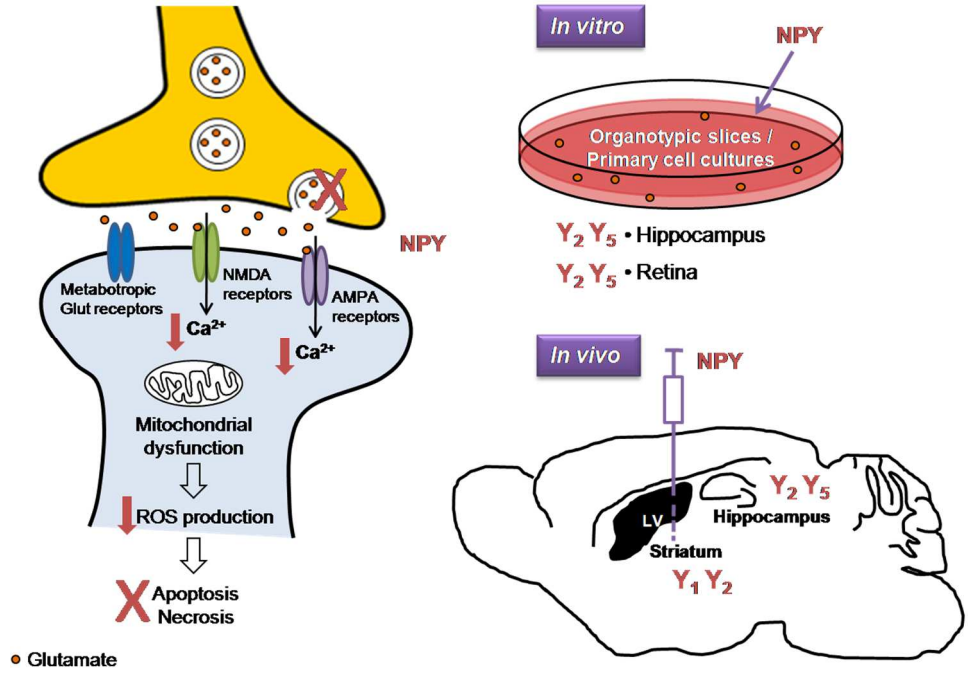


**Figure 1.6 – NPY decreases excitotoxicity and regulates calcium homeostasis.** Excitotoxicity is caused by prolonged or excessive activation of glutamate receptors that leads to a neuronal $Ca^{2+}$ overload, which can result in mitochondrial dysfunction, ROS production and ultimately neuronal death, either by apoptosis or necrosis. NPY may counteract excitotoxicity since NPY is able to block glutamate release, to decrease $Ca^{2+}$ levels, to inhibit ROS production and, thereby, to rescue neurons from apoptosis or necrosis. This NPY neuroprotective potential, through $Y_2$ and $Y_5$ receptors activation, was observed in hippocampal and retinal cell cultures, and also *in vivo*, in rodent hippocampus and striatum, mediated by $Y_2$ and $Y_5$ receptors and $Y_1$ and $Y_2$ receptors, respectively.

Taking this into account, NPY receptor activation can be a very relevant strategy to attenuate excitotoxicity linked to neurodegenerative disorders, since NPY is able to decrease glutamate effects in the rise of intracellular $Ca^{2+}$ levels, to modulate pathways of cellular death and to alleviate oxidative stress.

### 1.2.2.4. NPY attenuates neuroinflammation

Astrocytes in primary cells cultures of cortex and retina contain NPY, which is present both in cytoplasm and nucleus (Barnea *et al.*, 2001, Alvaro *et al.*, 2007); this NPY is secreted and is probably able to regulate transmitter release from nearby neurons but also astrocytes themselves, since NPY receptors are expressed in these glial cells (Ramamoorthy and Whim, 2008). The involvement of NPY in neuroinflammation is also supported by the fact that NPY and the $Y_1$ receptor levels are increased upon microglia activation, as shown in an endotoxin-mediated model of inflammation in a microglial cell line (Ferreira *et al.*, 2010), and that NPY expression is induced by the pro-inflammatory cytokine IL-1β (Barnea *et al.*, 2001). This NPY up-regulation may be a feedback mechanism to counteract the inflammatory process, since NPY is in turn able to inhibit the release of IL-1β and nitric oxide via activation of $Y_1$ receptors (Ferreira *et al.*, 2010). By attenuating these inflammatory signals, NPY inhibits phagocytosis promoted by microglia (Ferreira *et al.*, 2011) and also microglial motility (Ferreira *et al.*, 2012), again through the $Y_1$ receptor activation, possibly reducing the number of activated microglial cells in the injury site and thus restraining the exacerbation of the inflammatory response (Figure 1.7).

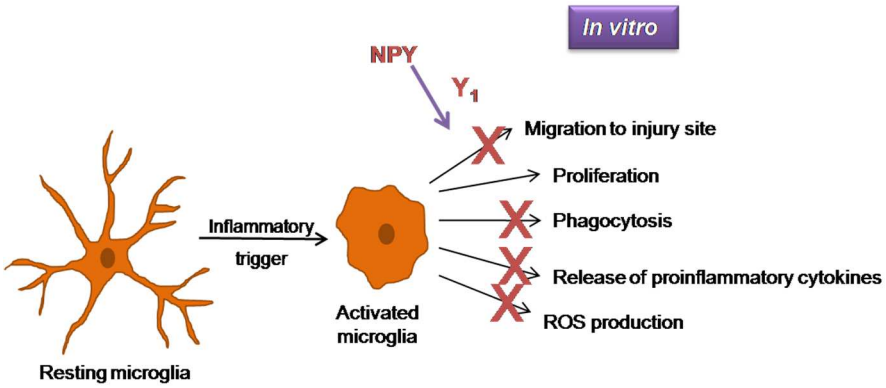


**Figure 1.7 – NPY attenuates neuroinflammation.** Upon CNS injury, microglia becomes activated and promotes an inflammatory response, which includes its migration to the site of injury, where it proliferates, secretes pro-inflammatory cytokines, stimulates ROS production and phagocytes damaged or invader cells, in an attempt to drive the resolution of the injury. In neurodegenerative diseases chronic inflammation can take place and microglia becomes overactivated and therefore harmful to CNS cells. *In vitro* studies indicate that NPY, through $Y_1$ receptor activation prevents microglial motility, inhibits phagocytosis and decreases proinflammatory cytokines and ROS production, restraining the exacerbation of the inflammatory response.

Another important feature of glial cells is their ability to clear the excess of glutamate from the extracellular space, protecting neurons against excitotoxicity. NPY can prevent microglial cell death induced by METH, although it also decreases the microglial response following this toxin exposure (Goncalves *et al.*, 2012), showing that NPY has a neuroprotective role also in these experimental conditions.

Therefore, NPY can reduce neuroinflammation and this effect might mediate neuroprotection.

#### 1.2.2.5. NPY counteracts depressive symptoms

Depression is very frequent in neurodegenerative disorders (Gotham *et al.*, 1986, Starkstein *et al.*, 2005, Cecchin *et al.*, 2007, Kingma *et al.*, 2008, Schmitz-Hubsch *et al.*, 2011, Fancellu *et al.*, 2013), increasing the impairment in everyday functioning and having a high impact in patients' quality of life (Kuopio *et al.*, 2000, Hamilton *et al.*, 2003, Starkstein *et al.*, 2005). It has been reported that in HD such psychiatric signs appear in gene carriers even before the onset of motor symptoms (Duff *et al.*, 2007, Kingma *et al.*, 2008). Since the etiology of PD and AD is not well known, it is difficult to evaluate if depression is an early symptom of the disorder or if it represents a risk factor for the development of the disease (Leentjens *et al.*, 2003, Ownby *et al.*, 2006, O'Sullivan *et al.*, 2008). Although one cannot dismiss psychosocial factors, like receiving the diagnosis of a chronic disabling disease and disability caused by the dementia and/or motor symptoms, or even side effects of anti-choreic drugs, like tetrabenazine (used by HD patients) (Huntington Study, 2006), they should not be solely accounted for the depression. In fact, depressive symptoms in neurodegenerative disorders can be due to several factors: impairment in the serotonergic and noradrenergic systems (Caraceni *et al.*, 1977, Chan-Palay and Asan, 1989, Reinikainen *et al.*, 1990, Cheng *et al.*, 1991, Steward *et al.*, 1993, Remy *et al.*, 2005, Kish *et al.*, 2008, Richards *et al.*, 2011), hypothalamic-pituitary-adrenal (HPA) axis dysfunction (Davis *et al.*, 1986, Masugi *et al.*, 1989, Gurevich *et al.*, 1990, Heuser *et al.*, 1991, Leblhuber *et al.*, 1995, Charlett *et al.*, 1998, Kassubek *et al.*, 2004, Politis *et al.*, 2008a, van Duijn *et al.*, 2010, Shirbin *et al.*, 2013), alterations in neurogenesis and in neurotrophic factor expression (reviewed in(Groves, 2007)) or neuroinflammation (Frommberger *et al.*, 1997, Nair and Bonneau, 2006, Pike and Irwin, 2006). Probably, all these factors act together promoting the depressive symptoms observed in neurodegenerative disorders.

NPY may be able to act in different mechanisms that are in the basis of depression, since it: increases the levels of serotonin (Redrobe *et al.*, 2005) and norepinephrine (Crespi, 2011); decreases HPA axis hyperactivity, as NPY can reduce plasma ACTH and cortisol plasma levels (Antonijevic *et al.*, 2000, Painsipp *et al.*, 2008a); stimulates neurogenesis

(Decressac *et al.*, 2009, Decressac *et al.*, 2011) and increments BDNF levels (Croce *et al.*, 2013), promoting survival of newborn neurons; and counteracts neuroinflammation, inhibiting the release of pro-inflammatory cytokines (Ferreira *et al.*, 2010) and attenuating the toxic effects of activated microglia (Ferreira *et al.*, 2011, Ferreira *et al.*, 2012).

Actually, several *in vivo* studies have shown that ICV NPY administration is able to produce antidepressive-like effects in rodents forced swimming test, a commonly used test for behavioral screening of potential antidepressant drugs, through the activation of $Y_1$ receptors (Stogner and Holmes, 2000, Redrobe *et al.*, 2002, Redrobe *et al.*, 2005), present in high density in stress related areas. Experiments using $Y_2$ receptors or $Y_4$ receptors knock-out (KO) mice revealed that the suppression of these receptors subtypes result in a significant restraint of depression (Tschenett *et al.*, 2003, Carvajal *et al.*, 2006, Painsipp *et al.*, 2008b, Tasan *et al.*, 2009); furthermore, the effects of $Y_2$ and $Y_4$ deletion may be cumulative, since the effects of $Y_2$/$Y_4$ double KO mice were more pronounced (Tasan *et al.*, 2009). Likewise, $Y_5$ receptors antagonism demonstrated to be a good strategy to reduce depression-related behavior in rodents (Walker *et al.*, 2009). Furthermore, in animal models of depression, despite some discrepancies between experimental designs and methodological procedures, antidepressants were able to enhance NPY levels in hippocampus, hypothalamus, cerebral cortex, amygdala, basal ganglia and *locus coerulus* (reviewed in (Obuchowicz *et al.*, 2004)), suggesting that antidepressant drugs may exert some of their therapeutic effects through up-regulation of endogenous NPY.

Classic antidepressant drugs, particularly fluoxetine, have proved to worsen the motor symptoms of HD and PD (Chari *et al.*, 2003, McDonald *et al.*, 2003). Furthermore, these drugs have a long therapeutic latency (2 to 3 weeks) and only 60-70% of depressed patients respond to these treatments (Kornstein and Schneider, 2001). Therefore, NPY can be a therapeutic alternative, playing a major role in attenuating depressive symptoms in neurodegenerative diseases.

#### 1.2.2.6. NPY increases food intake and body weight

Weight loss is frequently associated with neurodegenerative disorders aggravating its course (Aziz *et al.*, 2008), since it can contribute to both morbidity and mortality. Either AD patients (McKhann *et al.*, 1984, Gillette-Guyonnet *et al.*, 2000), PD patients (Beyer *et al.*, 1995, Chen *et al.*, 2003, van der Marck *et al.*, 2012), HD patients (Sanberg *et al.*, 1981, Farrer and Meaney, 1985, Trejo *et al.*, 2004) or MJD patients (Riess *et al.*, 2008, Saute *et al.*, 2012) have a significant loss of body weight and body mass index than matched controls from the general population. The loss of weight has been reported to begin several years before the formal diagnosis has been made (White *et al.*, 1997, Gillette-Guyonnet *et al.*, 2000, Djousse

*et al.*, 2002, Chen *et al.*, 2003, Buchman *et al.*, 2005, Johnson *et al.*, 2006) and is related to the stage of the disease – increased severity of the disease is associated with accentuation of weight loss (Beyer *et al.*, 1995, White *et al.*, 1998, Wang *et al.*, 2004, Guerin *et al.*, 2005, Saute *et al.*, 2012, Soto *et al.*, 2012) or higher body weight at the time of the diagnosis is associated with slower disease progression (Myers *et al.*, 1991).

The specific factors contributing to weight loss are not completely known, but probably it is multifactorial and related to increased energy expenditure and/or decreased caloric intake. Dyskinesias and dystonia can lead to higher energy expenditure in PD, HD and MJD, although they cannot be responsible for the weight loss in asymptomatic HD gene carriers or HD patients in early stages of the disease. Moreover, weight gain after pallidotomy and deep brain stimulation in PD patients is not associated with motor parameter changes (Ondo *et al.*, 2000, Macia *et al.*, 2004). Loss of appetite can be associated with the olfactory dysfunction that occurs early in the course of MJD, HD, PD and AD (Hawkes, 2003, Braga-Neto *et al.*, 2011) but also with the side-effects (nausea and anorexia) of some drugs that are in the first line of these diseases treatment, like acetylcholinesterase inhibitors and dopaminergic medication. Caloric intake can also be affected by dysphagia (Kagel and Leopold, 1992, Chouinard, 2000, Pfeiffer, 2003, Rub *et al.*, 2006a, Isono *et al.*, 2013) although this feature is only present in a later stage of the disease and thus cannot be indicted for the early weight loss.

NPY may be a valuable therapeutic agent in these neurodegenerative disorders, since it is an orexigenic agent (stimulates feeding), playing an important role in physiological control of food intake and body weight (Kalra *et al.*, 1991). In fact, central administration of NPY, either by ICV injection or directly injected in hypothalamic nuclei in rodent models, elicits a strong feeding response (Clark *et al.*, 1984, Levine and Morley, 1984, Stanley and Leibowitz, 1985, Sousa-Ferreira *et al.*, 2011). Moreover, fasting or food restriction increases hypothalamic NPY levels, either in rodent models (Brady *et al.*, 1990, Swart *et al.*, 2002, Bi *et al.*, 2003) or in eating disorders patients (Kaye *et al.*, 1990, Gendall *et al.*, 1999) representing a compensatory response to weight loss. Several studies using NPY receptors agonists, antagonists and knock-out rodent models brought to light that $Y_1$, $Y_2$ and $Y_5$ receptors can operate together to have orexigenic or anorexigenic effects: despite some controversial results, it is believed that $Y_1$ and $Y_5$ receptors mediate the stimulatory effect of NPY on food intake, while the $Y_2$ receptor has the opposite effect (reviewed in (Mercer *et al.*, 2011)).

For its important functions in energy homeostasis, NPY can be of major importance for individuals suffering from neurodegenerative diseases.

### 1.3. Viral-mediated gene delivery to the CNS: disease modeling and therapeutic approaches

Neurodegenerative disorders can be modeled in several mammalian species through stereotaxic delivery of viral vectors in specific brain regions (reviewed in (Deglon and Hantraye, 2005)). This strategy has proved to be very useful in elucidating and dissecting the molecular basis of these diseases. Indeed, several neuropathological features and behavioral abnormalities were obtained by overexpression of the disease-causing protein creating valuable models of PD (Kirik *et al.*, 2002, Lo Bianco *et al.*, 2002, Kirik *et al.*, 2003, Lauwers *et al.*, 2003), HD (de Almeida *et al.*, 2002, DiFiglia *et al.*, 2007, Palfi *et al.*, 2007) and MJD (Alves *et al.*, 2008b, Simoes *et al.*, 2012, Nobrega *et al.*, 2013a). These genetic viral-mediated vectors hold various advantages compared with classical transgenic approaches: i) the onset and time-course of the degeneration can be controlled; ii) the generation of the disease model is possible in a short period of time, due to high transduction efficiencies and to robust and sustained transgene expression; iii) viral vectors can be introduced in specific brain regions of the brain allowing a selection of the brain regions affected by the disease, eliminating unexpected phenotypic effects associated with a widespread overexpression of the transgene; iv) expression levels of the disease causing-protein can be controlled by manipulating the titer and/or amounts of virus injected; v) these models can be established in different mammalian species (Senut *et al.*, 2000, Deglon and Hantraye, 2005).

Furthermore, gene delivery system based in viral vectors have proved to be promising for evaluating the therapeutic potential of disease-modifier genes (Kordower *et al.*, 2000, de Almeida *et al.*, 2001, Nascimento-Ferreira *et al.*, 2011, Simoes *et al.*, 2012, Goncalves *et al.*, 2013, Nascimento-Ferreira *et al.*, 2013). Moreover, viral vectors have also been used to deliver RNA interference mediators to allow the shutting-down of the disease-causing gene (Xia *et al.*, 2004, Harper *et al.*, 2005, Alves *et al.*, 2008a, Franich *et al.*, 2008, Alves *et al.*, 2010, Nobrega *et al.*, 2013b, Rodriguez-Lebron *et al.*, 2013, Nobrega *et al.*, 2014).

Due to the ability to cross cellular membranes and to deliver genetic material to host cells with very high efficiency, viral vectors have been widely used for CNS applications, being by far the most popular delivery system used in gene therapy clinical trials (Figure 1.8; reviewed in (Ginn *et al.*, 2013)).

There is a wide range of viral vectors (Table 1.8) and since each disease, therapeutic gene and brain region has special features, the choice of vectors of delivery needs to be tailored accordingly (reviewed in (Costantini *et al.*, 2000, Thomas *et al.*, 2003, Nobre and Almeida, 2011)). Accordingly, each one of these vectors present specific advantages based on its packaging capacity, its ability or not to integrate into the host genome, its cell or tissue

tropism and its tendency to elicit or not immune responses. For neurodegenerative diseases modeling and therapeutic strategies assessment, viral vectors should promote a sustained and long term expression of the transgene with low immunogenicity.

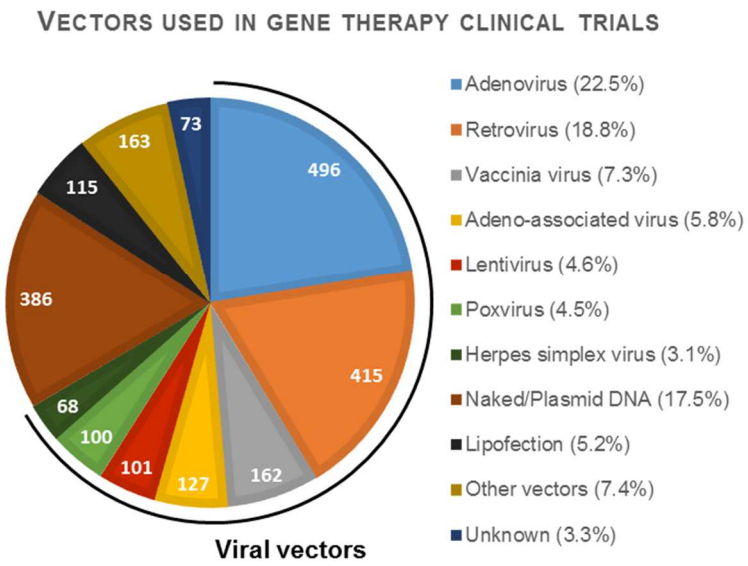


**Figure 1.8 – Vectors used in gene therapy clinical trials.** Adapted from (Ginn *et al.*, 2013)

**Table 1.8 – The main groups of gene transfer viral vectors.**

| Vector | Packaging capacity | Tropism | Inflammatory potential | Vector genome forms |
|---|---|---|---|---|
| Retroviral vectors | 8 Kb | Only dividing cells | Low | Integrated |
| Lentiviral (LV) vectors | 8 Kb | Broad | Low | Integrated |
| Herpes simplex virus-1 (HSV-1) | 180 Kb | Strong for neurons | High | Episomal |
| Adeno-associated viral (AAV) vectors | < 5 Kb | Broad | Low | Predominantly episomal |
| Adenoviral vectors | 30 Kb | Broad | High | Episomal |

Adapted from (Thomas *et al.*, 2003).

## 1.4. Objectives

The main aim of this work was to investigate the potential NPY role in prevention or reduction of MJD neuropathology, in an attempt to contribute to the development of a therapeutic strategy for MJD.

The specific objectives of this thesis were:

- to evaluate NPY levels in MJD patients postmortem tissue and MJD mouse models (chapter 2, 3 and 4);
- to investigate whether AAV-mediated NPY overexpression in a lentiviral-based mouse model of MJD reduces mutant ataxin-3 inclusions and associated neuronal dysfunction (chapter 2);
- to study if AAV-mediated NPY overexpression alleviates the behavioral deficits of a transgenic MJD mouse model (chapter 3);
- to investigate if hypothalamus, a brain region with high levels of NPY, is affected in MJD transgenic mouse models (chapter 4).

# CHAPTER 2

## Neuropeptide Y overexpression mitigates striatal neuropathology in a lentiviral-based mouse model of Machado-Joseph disease

## 2.1 Introduction

Polyglutamine (polyQ) diseases are characterized by a pathologic expansion of the CAG trinucleotide repeats which translate into an abnormally elongated polyQ chain in the affected protein (Shao and Diamond, 2007). There are at least 9 polyQ disorders, including Machado-Joseph disease (MJD), also known as spinocerebellar ataxia type 3 (SCA3). Indeed, MJD is caused by an over-repetition of the CAG trinucleotide in the coding region of the *ATXN3* gene that encodes an expanded polyglutamine stretch in the corresponding protein, ataxin-3 (Kawaguchi *et al.*, 1994). This protein carries a C-terminal polyglutamine tract; when the number of repeated glutamines exceeds 61, the protein becomes toxic, adopts an abnormal folding and has an increased propensity to aggregate producing intranuclear inclusion bodies (Paulson *et al.*, 1997b, Maciel *et al.*, 2001). The expression of mutant ataxin-3 leads to neuronal dysfunction and degeneration in specific regions of the CNS, such as cerebellum, *substantia nigra* and striatum (Sudarsky and Coutinho, 1995, Durr *et al.*, 1996, Klockgether *et al.*, 1998). This neurodegeneration is responsible for progressive ataxia, dystonia, peripheral neuropathy and oculomotor abnormalities (Paulson, 2007, D'Abreu *et al.*, 2010, Nóbrega and Pereira de Almeida, 2012).

The exact pathogenic mechanisms that lead to neurodegeneration in MJD are not completely understood, but it has been shown that excitotoxicity (Chen *et al.*, 2008, Koch *et al.*, 2011), proteolysis (Simoes *et al.*, 2012, Simoes *et al.*, 2014), transcriptional dysfunction (Chou *et al.*, 2008), autophagy impairments (Nascimento-Ferreira *et al.*, 2011, Nascimento-Ferreira *et al.*, 2013) and neuroinflammation (Evert *et al.*, 2001, Silva-Fernandes *et al.*, 2010, Goncalves *et al.*, 2013) contribute to the pathogenesis. MJD is a fatal disease and so far there are no therapies available to prevent or stop the progression of the disorder.

Neuropeptide Y (NPY) is a 36-aminoacid peptide that is abundantly and unequally distributed in several mammalian brain regions. It is involved in many physiological functions, through the activation of NPY receptors, $Y_1$, $Y_2$, $Y_4$ and $Y_5$ receptors (Silva *et al.*, 2005). It has been shown that NPY inhibits neural cell death (Santos-Carvalho *et al.*, 2013), increases trophic support (Xapelli *et al.*, 2008, Croce *et al.*, 2013), stimulates autophagy in hypothalamic neurons (Aveleira *et al.*, 2015), exhibits anti-inflammatory effects (Ferreira *et al.*, 2010, Ferreira *et al.*, 2012) and overall is a potent neuroprotective molecule (Silva *et al.*, 2005, Alvaro *et al.*, 2008b, Decressac *et al.*, 2010, Decressac *et al.*, 2012).

Therefore, the first aim of the present study was to evaluate NPY levels in the striatal lentiviral-based MJD mouse model. Since we observed a decrease of NPY in the analyzed samples, we hypothesized that the increase of NPY in the striatum of this MJD model could have an impact on the progression of the disease. Therefore, taking advantage of AAV vectors, we evaluated whether NPY overexpression would mitigate the MJD-associated neuropathology. For the first time, we provide evidence that NPY overexpression is able to

significantly alleviate MJD-related neuropathology, which may be associated to the increase of brain-derived neurotrophic factor (BDNF) and the attenuation on neuroinflammation.

## 2.2. Materials and methods

### 2.2.1. Animals

Young adult male C57BL/6J mice (Charles River, Spain) with 5-7 weeks of age were used in this study.

The experiments were carried out in accordance with the European Community directive (2010/63/EU) covering the protection of animals used for scientific purposes. The researchers received adequate training (FELASA-certified course) and certification to perform the experiments from the Portuguese authorities (Direcção Geral de Alimentação e Veterinária).

### 2.2.2. Viral vectors production

Lentiviral vectors encoding human wild-type ataxin-3 (Atx3WT) with 27 glutamines or mutant ataxin-3 (Atx3MUT) with 72 glutamines (Alves *et al.*, 2008b), under the control of phosphoglycerate kinase (PGK) promoter, were produced in HEK-293T cells with a four-plasmid system, as previously described (de Almeida *et al.*, 2001). The lentiviral particles were resuspended in 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS). The viral particle content of batches was determined by assessing HIV-1 p24 antigen levels (RETROTek, Gentaur, France). Viral stocks were stored at -80ºC.

AAV serotype 1/2 vectors encoding NPY or enhanced green fluorescent protein (EGFP) under the control of human synapsin promoter (Sousa-Ferreira *et al.*, 2011) were used.

### 2.2.3. *In vivo* injection of viral vectors into mice striatum

After anesthesia of the mice with Avertin (280 µg/g, intraperitoneally), viral vectors were stereotaxically injected into the striatum at the following coordinates: anteroposterior +0.6 mm, lateral ±1.8 mm and ventral -3.3 mm, relative to bregma (Paxinos *et al.*, 2001).

For lentiviral-based MJD model, wild-type mice received a single 2 µl injection of lentivirus (200000 ng of p24/ml) in each hemisphere, encoding Atx3MUT in the right hemisphere and Atx3WT in the left hemisphere, as internal control. For evaluation of neuroprotection in the striatal model, wild-type animals were bilaterally co-injected with 1 µl of Atx3MUT lentivirus (400000 ng of p24/ml) and 2.5 µl AAV-1/2-NPY or AAV-1/2-EGFP

($0.8x10^8$ t.u.) as control. After the injection, the syringe was left in place for an additional 5 minutes to allow the diffusion of the viral vectors and minimize backflow.

### 2.2.4. Immunohistochemical procedure

After an overdose of Avertin (2.5 x 280 µg/g intraperitoneally), transcardial perfusion of the mice was performed with PBS followed by fixation with 4% paraformaldehyde (PFA; Sigma-Aldrich, USA). The brains were removed and post-fixed in 4% PFA for 24h and cryoprotected by incubation in 25% sucrose/PBS for 48 h. The brains were frozen and 25 µm coronal sections were cut using a cryostat (LEICA CM3050S, Germany) at -21ºC. Sections throughout the entire striatum were collected in anatomical series and stored in 48-well trays as free-floating sections in PBS supplemented with 0.05 µM sodium azide (Sigma-Aldrich), at 4ºC.

The immunohistochemical procedure was initiated by endogenous peroxidase quenching with 30 min incubation at 37ºC in 0.1% phenylhydrazine (Merck, USA) /PBS solution. Sections were then kept at room temperature (RT) for 1 h in blocking solution constituted by PBS with 0.1% Triton X-100 (Sigma-Aldrich) containing 10% goat serum (NGS; Gibco, Alfagene, Portugal), then overnight at 4ºC in blocking solution with the primary antibodies: a mouse monoclonal anti-ataxin-3 antibody (1H9; 1:5000; Chemicon, Merck-Millipore, USA), a rabbit polyclonal anti-ubiquitin antibody (1:1000; Dako, Denmark) or a rabbit anti-dopamine- and cyclic AMP-regulated phosphoprotein of 32 kDa (DARPP-32; 1:1000; Chemicon, Merck-Millipore). After washing, the sections were incubated with the respective biotinylated secondary antibodies (1:200; Vector Laboratories, USA). Bound antibodies were visualized using the VECTASTAIN® ABC kit, with 3,3'-diaminobenzidine tetrahydrochloride (DAB metal concentrate; Pierce, USA) as substrate. The sections were mounted, dehydrated and coverslipped with Eukitt® (Sigma-Aldrich).

Double staining for NPY (rabbit anti-NPY; 1:6000; Sigma-Aldrich), Ionized calcium-binding adapter molecule 1 (Iba1; rabbit anti-Iba1; 1:1000; Wako, Germany) and nuclear marker [4',6-diamidino-2-phenylindole (DAPI); Sigma-Aldrich] were performed. After RT 1 h incubation with blocking solution, and overnight 4ºC incubation with primary antibodies, sections were washed and incubated for 2 h at RT with the corresponding secondary antibodies coupled to fluorophores (1:200; Molecular Probes, Life Technologies, USA) diluted in the respective blocking solution. The sections were then mounted in Mowiol reagent (Sigma-Aldrich) on microscope slides.

Staining was visualized using Zeiss Axioskop 2 plus imaging microscope (Carl Zeiss Microimaging, Germany) using 5x and 20x objectives and the AxioVision 4.7 software package (Carl Zeiss Microimaging).

Quantitative analysis was performed with a semiautomated image-analysis software package (ImageJ 1.42q software, USA).

### 2.2.5. Cell counts of striatal NPY-positive interneurons and mutant ataxin-3 inclusions

Twelve coronal sections at 200 µm intervals that encompassed the entire region transduced by the viral vectors were scanned with a 20x objective. All striatal NPY-positive interneurons and mutant ataxin-3 inclusions were manually counted, and its number extrapolated for the entire striatum.

### 2.2.6. Evaluation of the DARPP-32 depleted volume

The extension of ataxin-3 lesions in the striatum was analyzed by photographing, with a 5x objective, 12 DARPP-32-stained sections per animal (25-µm-thick-sections at 200 µm intervals), selected to obtain complete rostrocaudal sampling of the striatum, and by quantifying the area of the lesion. The volume was then estimated with the following formula: volume = $d(a_1 + a_2 + a_3 + \ldots)$, where $d$ is the distance between serial sections (200 µm) and $a_1 + a_2 + a_3$ are DARPP-32 depleted areas for individual serial sections (de Almeida *et al.*, 2002).

### 2.2.7. Quantification of Iba1 immunoreactivity

The Iba1 immunoreactivity indexes were measured through optic density analysis of the affected striatal regions relative to their corresponding non-affected cortex (defined as background).

### 2.2.8. Western blot analysis

After an overdose of Avertin, mice were sacrificed by cervical dislocation and injected striata were dissected and immediately sonicated in radioimmunoprecipitation assay (RIPA) buffer [50 mM Tris-HCl pH 8, 150 mM NaCl, 1% nonyl phenoxypolyethoxylethanol (NP-40), 0.5% sodium deoxycholate, 0.1% Sodium dodecyl sulphate (SDS), 10 µg/mL dithiothreitol (DTT; Sigma-Aldrich), 1 mM phenylmethylsulphonyl fluoride (PMSF; Sigma-Aldrich), protease inhibitors cocktail (Roche, Switzerland)]. Equal amounts of protein were resolved

on 12% SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF; Merck-Millipore) membranes. Immunoblotting was performed using 1H9 (1:2000; Chemicon, Merck-Millipore), anti-NPY antibody (1:1000; Sigma-Aldrich), anti-BDNF antibody (1:200, Alomone Labs, Israel) and anti-actin (clone AC-74; 1:5000; Sigma-Aldrich) antibodies. A partition ratio with actin was calculated following quantification with Quantity One 1-D Image analysis software version 4.6.8 (Bio-Rad, USA).

### 2.2.9. Isolation of mRNA and cDNA synthesis

After an overdose of Avertin mice were sacrificed by cervical dislocation and injected striata were dissected and stored in tubes containing RNAlater RNA stabilization reagent (QIAGEN, Germany) at -80°C until extraction of RNA. Total RNA was isolated using the Nucleospin RNA Isolation Kit (Macherey-Nagel, Germany) according to the manufacturer's instructions. Total amount of RNA was quantified using a Nanodrop 2000 Spectrophotometer (Thermo Scientific, USA) and the purity was evaluated by measuring the ratio of optical density (OD) at 260 and 280 nm. cDNA was then obtained by conversion of 1 µg of total RNA using iScript Selected cDNA Synthesis kit (Bio-Rad) according to the manufacturer's instructions and stored at -20ºC.

### 2.2.10. Quantitative real-time polymerase chain reaction (qRT-PCR)

Quantitative PCR was performed in a StepOnePlus Real-Time PCR system (Applied Biosystems). The primers for the mouse genes – *Npy*, *Bdnf*, *Il6*, *Il1b*, *Tnfa*, *Hprt* and *Gapdh* – were pre-designed and validated by QIAGEN (QuantiTect Primers, QIAGEN). A master mix was prepared for each primer set containing the appropriate volume of SsoAdvanced SYBR Green Supermix (Bio-Rad), QuantiTect Primers and template cDNA. All reactions were performed in duplicate and according to the manufacturer's recommendations: 95ºC for 30 s, followed by 40 cycles at 95ºC for 5 s and 60ºC for 30 s. The melting curve protocol started immediately after amplification. The amplification efficiency for each primer pair and the threshold cycle (Ct) were determined automatically by StepOne Software (Applied Biosystems). The mRNA fold change with respect to control samples was determined by the Pfaffl method, taking into consideration amplification efficiencies of all genes.

### 2.2.11. Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Statistical comparisons were performed using either paired or unpaired Student's t-test or two-way ANOVA followed by Bonferroni *post hoc* test. Significance thresholds were set at $p<0.05$, $p<0.01$ or $p<0.001$.

## 2.3. Results

### 2.3.1. Striatal NPY levels are decreased in a mouse model of MJD

In order to investigate NPY levels in MJD, we analyzed samples from striatal lentiviral-based mouse model of the disease.

In the striatal MJD mouse model, lentiviral vectors encoding Atx3MUT were injected in one striatal hemisphere and lentivirus encoding Atx3WT in the other, as control, and 4 weeks post-injection mice were sacrificed. The number of striatal positive NPY-expressing interneurons (Fig. 2.1A and B) in the mutant ataxin-3 transduced hemisphere was significantly reduced compared with Atx3WT expressing hemisphere (4848 ± 417 versus 5709 ± 374 control, * $p<0.05$, n=5, Fig. 2.1C). Indeed, Atx3MUT expression significantly decreased (* $p<0.05$, n=4) the *Npy* mRNA levels when compared with the control hemisphere (Fig. 2.1D).

These results show that mutant ataxin-3 expression reduces striatal NPY levels in MJD mouse model.

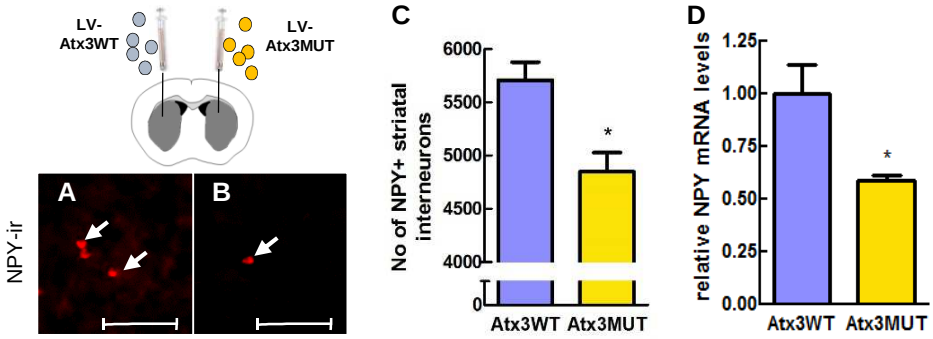


**Figure 2.1 –** NPY levels are reduced in the striatal lentiviral mouse model of MJD. Four-week-old mice were injected in the striatum with lentiviral vectors encoding for Atx3MUT (right hemisphere) and for Atx3WT (left hemisphere) as internal control, and were sacrificed 4 weeks post-injection. (**A** and **B**) Fluorescence staining of NPY. The number of striatal NPY-positive interneurons was decreased (* $p<0.05$, n=5) where mutant ataxin-3 was expressed, as quantified in **C**. (**D**) Atx3MUT overexpression decreased (* $p<0.05$, n=4) by 42% *Npy* mRNA levels. Statistical significance was evaluated with paired Student's t-test. Data are expressed as mean ± SEM. Scale bars: 100µm.

### 2.3.2. Experimental strategy used to overexpress NPY in the striatum of lentiviral-based mouse model of MJD.

Since NPY levels are reduced in the striatal lentiviral mouse model of the disease, we aimed at evaluating NPY positive effects in MJD-associated neuropathology by overexpressing NPY in the affected brain area of these mice.

For that, we bilaterally co-transduced the striatum of 5-week-old mice with LVs encoding for Atx3MUT and AAVs encoding for NPY or for EGFP, as control (Fig. 2.2A). AAV vectors mediate a delayed expression of NPY, when compared to a quicker onset of ataxin-3 expression upon lentiviral transduction, due to the necessity of conversion of the single stranded genome into double stranded DNA, especially in non-dividing cells (Shevtsova *et al.*, 2005).

AAV-NPY injection induced a 31-fold increase (** p<0.01, n=4) in *Npy* mRNA striatal levels (Fig. 2.2B) promoting a higher striatal NPY-immunoreactivity (Fig. 2.2C and D) further confirmed by western blot analysis (Fig. 2.2E).

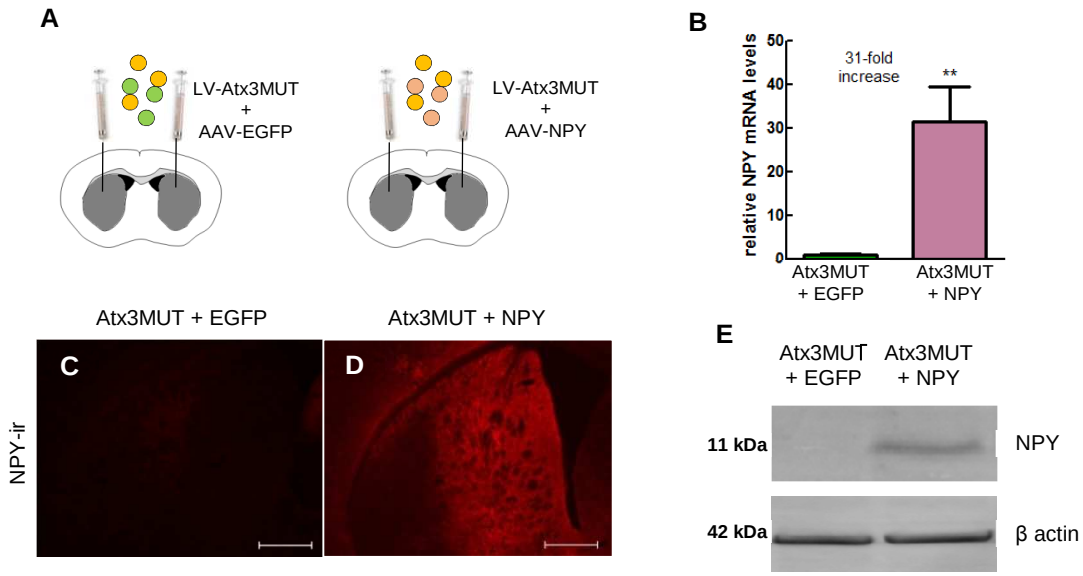


**Figure 2.2 –** AAVs promoted a robust NPY overexpression in mice striata. (**A**) Five-week-old mice were bilaterally co-injected in the striatum with viral vectors encoding for Atx3MUT and for NPY or for EGFP, as control. (**B**) Quantitative PCR analysis revealed a 31-fold increase (** p<0.01, n=4) of *Npy* expression achieved by the striatal delivery of viral vectors encoding NPY. (**C**-**D**) Fluorescence staining for NPY and (**E**) western blot analysis with anti-NPY antibody, showing that the stereotaxic injection of the viral vectors allowed the overexpression of NPY. Statistical significance was evaluated with unpaired Student's t-test. Data are expressed as mean ± SEM. Scale bars: 500 μm.

### 2.3.3. NPY overexpression reduces the number of mutant ataxin-3 inclusions.

Using this mouse model we evaluated NPY overexpression in the formation of mutant ataxin-3 aggregates.

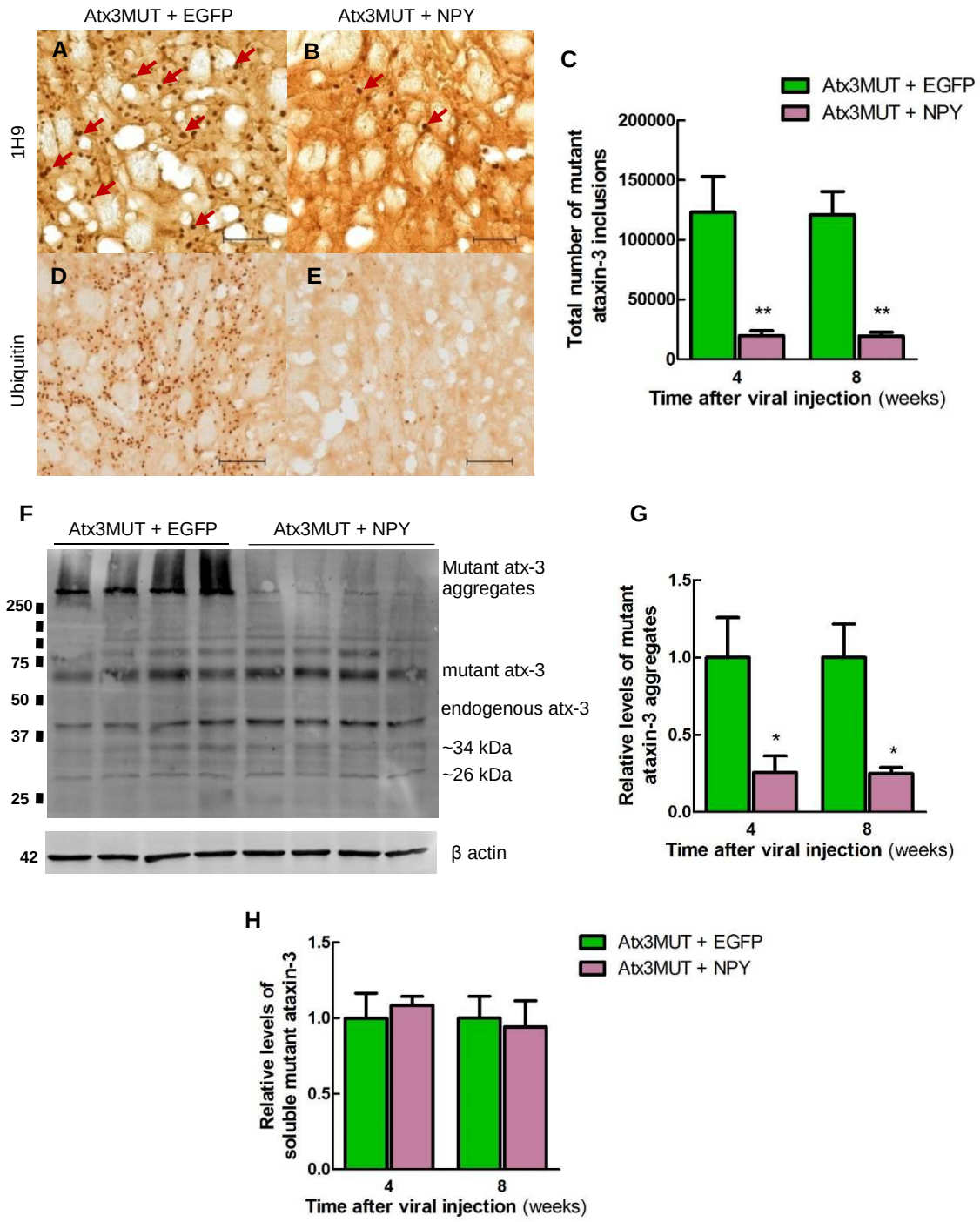


**Figure 2.3 –** NPY overexpression significantly reduces the number of mutant ataxin-3 inclusions. (**A**-**B**) Immunohistochemical peroxidase staining using anti-ataxin3 antibody (1H9 ab), 8 weeks post-injection. Control Atx3MUT+EGFP-injected animals displayed a large number of mutant ataxin-3 inclusions (arrows, **A**) which was significantly decreased (** $p<0.01$, $n=4$) in the NPY-transduced striatum (**B**) both at 4 and 8 weeks post-injection, as quantified in **C**. (**D** and **E**) Co-

transduced striatum with control EGFP also revealed a large number of ubiquitin-positive inclusions (**D**), which were almost absent in the NPY-transduced striatum (**E**). (**F**) Western blot (1H9 ab) of transduced striata allows the detection of different forms of ataxin-3: mutant ataxin-3 aggregates (that accumulated in the stacking gel), the soluble mutant ataxin-3, the endogenous ataxin-3 and the two fragments (~34 and ~26 kDa) resulting from proteolytic cleavage of the mutated protein. The western blot analysis revealed a clear aggregation pattern of mutant ataxin-3 in control EGFP striata after viral transduction, which were significantly reduced (* $p<0.05$, n=4) in NPY co-transduced striatal hemispheres, at both 4 and 8 weeks, as quantified in **G**. (**H**) NPY overexpression did not change soluble mutant ataxin-3 levels (n=4). Statistical significance was evaluated with two-way ANOVA with Bonferroni *post hoc* test. Data are expressed as mean ± SEM. Scale bars: 100 µm.

NPY overexpression in the MJD striata robustly decreased the number of mutant ataxin-3 neuronal inclusions (Fig. 2.3B) when compared with control EGFP co-transduction (Fig. 2.3A), either at 4 weeks (19820 ± 4080 versus 123000 ± 29910 control, ** $p<0.01$, n=4) and at 8 weeks post-injection (19480 ± 3080 versus 120900 ± 19640 control, * $p<0.01$, n=4; Fig. 2.3C). Additionally, a large number of ubiquitin-positive inclusions was observed in the control animals (co-injected with mutant ataxin-3 and EGFP, Fig. 2.3D), which were almost absent in the NPY co-transduced striata (Fig. 2.3E). The observed reduction of mutant ataxin-3 inclusions induced by NPY overexpression was further confirmed by western blot analysis of the striatal total extracts (Fig. 2.3F), which revealed a 75% decrease of immunolabelled mutant ataxin-3 aggregates, at both 4 and 8 weeks post-injection of viral vectors (* $p<0.05$, n=4, Fig. 2.3G), when compared with the control mice. No changes were observed in the soluble mutant ataxin-3 levels between the two experimental groups (Fig. 2.3H).

These results show that NPY overexpression prevents the accumulation of mutant ataxin-3 neuronal inclusions in the lentiviral striatal MJD model.

### 2.3.4. NPY overexpression induces striatal neuroprotection.

To investigate whether NPY overexpression would prevent neuronal dysfunction induced by mutant ataxin-3, we performed immunohistochemical detection of DARPP-32, an intracellular regulator of the dopaminergic signaling (Greengard *et al.*, 1999). This marker was previously shown to be decreased upon early neuronal dysfunction (de Almeida *et al.*, 2002, Alves *et al.*, 2008b, Simoes *et al.*, 2012, Goncalves *et al.*, 2013). Indeed, mutant ataxin-3 expression mediated a striatal functional damage typified by a large volume of DARPP-32 immunoreactivity loss over time (Fig. 2.4A and C), which was reduced by over 55% upon

NPY overexpression (Fig. 2.4B and D), both 4 weeks (** p<0.01, n=4) and 8 weeks post-injection (* p<0.05, n=4), as shown in Fig. 2.4E.

These data indicate that NPY reduces striatal neuronal dysfunction in this animal model of MJD.

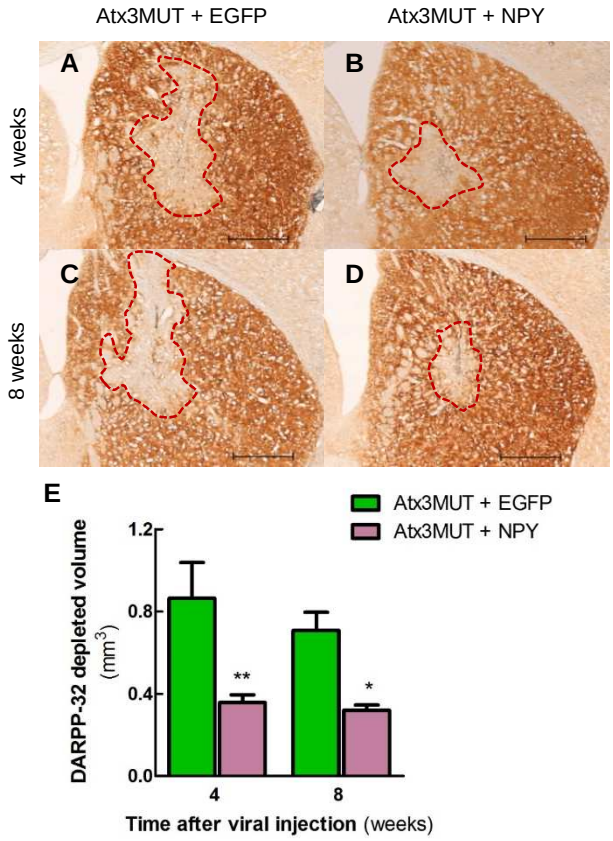


**Figure 2.4 –** NPY overexpression induces striatal neuroprotection. (**A**-**D**) Immunohistochemical peroxidase staining using an anti-DARPP-32 antibody. (**A** and **C**) Striatal co-transduction of mutant ataxin-3 and EGFP induced a striatal lesion characterized by depletion of DARPP-32 immunoreactivity, 4 and 8 weeks post-injection. (**B** and **D**) NPY overexpression significantly reduced the loss of DARPP-32 staining volume, at 4 and 8 weeks post-injection, as quantified in **E** (** p<0.01 and * p<0.05, respectively, n=4). Statistical significance was evaluated with two-way ANOVA followed by Bonferroni *post hoc* test. Data are expressed as mean ± SEM. Scale bars: 500 µm.

### 2.3.5. NPY overexpression up-regulates BDNF in MJD striatum.

It has been previously demonstrated that BDNF levels are decreased in neurodegenerative disorders (Ferrer *et al.*, 2000, Hock *et al.*, 2000), and that BDNF overexpression is neuroprotective (Xie *et al.*, 2010b).

To assess whether NPY neuroprotective effects were related to an induction of BDNF up-regulation, we assessed *Bdnf* mRNA levels in the striatal rodent model of MJD. We found that NPY overexpression mediated a 6.5 times increase of the *Bdnf* mRNA levels in striata transduced with mutant ataxin-3 (* p<0.05, n=4), 4 weeks post-injection, compared with the control EGFP co-transduction (Fig. 2.5A). By western blot analysis, we also observed an increase of BDNF protein levels (** p<0.01, n=4) in Atx3MUT-transduced striata (Fig. 2.5B).

These results indicate that NPY overexpression promotes production of trophic support for striatal neurons in this mouse model of MJD.

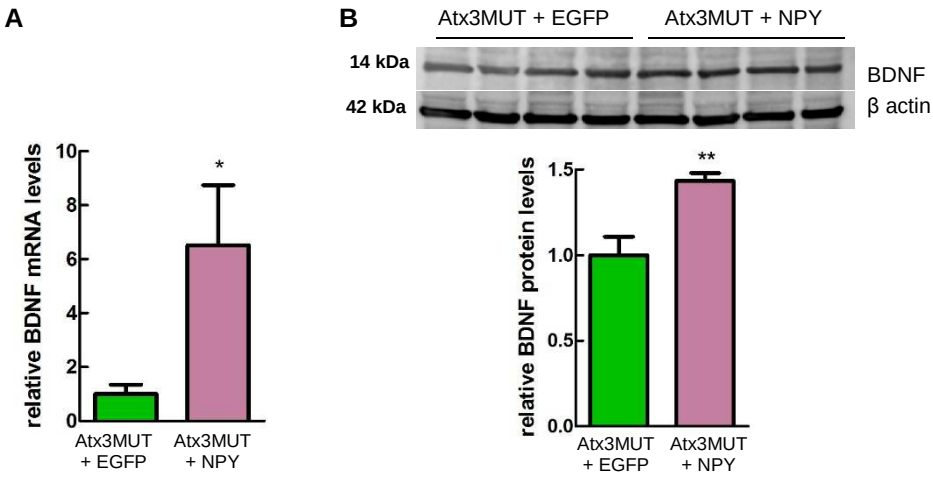


**Figure 2.5 –** NPY overexpression induces BDNF up-regulation. (**A**) Quantitative PCR analysis showed that NPY overexpression induced a 6.5 fold increase of the *Bdnf* mRNA levels in the Atx3MUT-transduced striata (* p<0.05 n=4), 4 weeks post-injection. (**B**) Western blot analysis revealed that NPY overexpression increases BDNF protein levels in the Atx3MUT striata (** p<0.01, n=4). Statistical significance was evaluated with unpaired Student's t-test. Data are expressed as mean ± SEM.

### 2.3.6. NPY reduces neuroinflammation in the striatal model of MJD.

Considering that neuroinflammation has been argued to be one of the pathogenic mechanisms in MJD (Evert *et al.*, 2001, Silva-Fernandes *et al.*, 2010, Goncalves *et al.*, 2013), we investigated the anti-inflammatory effect of NPY overexpression in the striatal model.

Strong immunoreactivity for Iba1 was detected in the injected striata (Fig. 2.6A and B), reveling microglia activation in the lesion site. Eight weeks post-injection, NPY overexpression induced a 31% decrease of Iba1 immunoreactivity relative to control Atx3MUT+EGFP (Fig. 2.6C, * p<0.05, n=4). Additionally, NPY overexpression reduced

striatal mRNA levels of proinflammatory cytokines such as *Il6* (Fig. 2.6D, * p<0.05, n=4), 4 weeks after vectors injection, when compared to control EGFP-injected mice.

Altogether, these results indicate that NPY overexpression attenuates neuroinflammation associated with MJD.

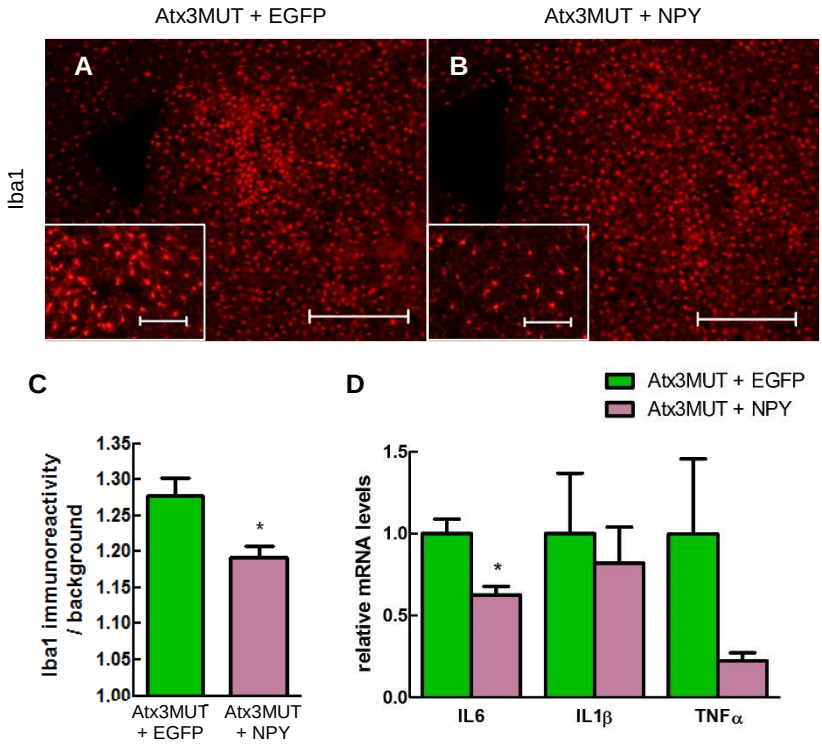


**Figure 2.6 –** NPY overexpression reduces neuroinflammation in MJD striatal model. (**A** and **B**) Fluorescence staining for ionized calcium-binding adapter molecule 1 (Iba1), a microglia marker. Striatal co-transduction of Atx3MUT+EGFP induced microglia activation gauged by enhanced Iba1 immunoreactivity, which was increased at 8 weeks post-injection. NPY overexpression prevented the increase of microglia activation at this time-point (* p<0.05, n=4), as quantified in (**C**). (**D**) Furthermore, NPY overexpression decreased mRNA levels of proinflammatory cytokine *Il6* (* p<0.05, n=4) at 4 weeks post-injection. Statistical significance was evaluated with unpaired Student's t-test. Data are expressed as mean ± SEM. Scale bars: 500 μm and 100 μm (higher magnifications).

## 2.4. Discussion

In this study, we showed, for the first time, that NPY levels are decreased in the striatal MJD lentiviral-based model and that NPY overexpression is able to alleviate MJD-related neuropathology in the same mouse model.

The striatal lentiviral-based rodent model of the disease, which enables the expression of a full-length mutated ataxin-3 in the striatum, a brain region also affected in MJD, allows precise, quantitative analysis of neuropathological deficits induced by mutant ataxin-3 expression (Alves *et al.*, 2008b, Alves *et al.*, 2010, Nascimento-Ferreira *et al.*, 2011, Simoes *et al.*, 2012, Goncalves *et al.*, 2013).

We found that *Npy* levels were reduced in the striata of the lentiviral-based model. Moreover, this reduction of *Npy* levels in the striatum is correlated with a reduction in the number of striatal NPY-positive interneurons. Hence, the described striatal neurodegeneration in MJD (Taniwaki *et al.*, 1997, Klockgether *et al.*, 1998, Alves *et al.*, 2008b) may also affect the NPY-positive neurons, causing the observed decrease of NPY levels.

NPY overexpression in the striatal model also mediated neuroprotection typified by a robust decrease in number of mutant ataxin-3 inclusions and reduction of DARPP-32 striatal lesion. The observed neuroprotective role of NPY is in accordance with previous reports in other paradigms (Alvaro *et al.*, 2008b, Xapelli *et al.*, 2008, Santos-Carvalho *et al.*, 2013) and neurodegenerative diseases (Decressac *et al.*, 2010, Decressac *et al.*, 2012, Croce *et al.*, 2013), namely in HD transgenic mice where an ICV injection of NPY reduced striatal atrophy (Decressac *et al.*, 2010), and in a rodent model of PD, in which a striatal injection of NPY preserved the nigrostriatal dopaminergic pathway (Decressac *et al.*, 2012).

To clarify the putative mechanisms through which NPY exerts its protective effects, we investigated its role in promoting BDNF trophic support and anti-inflammatory effects. The increase of BDNF levels induced by NPY overexpression may contribute to its neuroprotective effect observed in MJD mouse models. BDNF is a neurotrophic factor and an important pro-survival agent that is depleted in some neurodegenerative diseases, including HD (Ferrer *et al.*, 2000) and AD (Hock *et al.*, 2000), and its overexpression in the forebrain of an HD mouse model prevented the loss of striatal neurons with subsequent rescue of motor phenotype (Xie *et al.*, 2010b). Moreover, Croce and colleagues showed that NPY was able to decrease amyloid β-peptide toxicity in an in vitro model of AD, through the up-regulation of BDNF, as a consequence of decreasing the microRNA (miR) 30a-5p (Croce *et al.*, 2013), previously shown to down-regulate BDNF (Mellios *et al.*, 2008). Thus, it is possible that NPY exerts neuroprotective effects in MJD by controlling miR expression and hence BDNF levels. On the other hand, the infusion of NPY into the rat hypothalamus leads to increased phosphorylation of CREB, an important transcription factor with multiple targets

(Sheriff *et al.*, 1997). Since BDNF and its receptor trkB are CREB-target genes (Tao *et al.*, 1998), we alternatively hypothesize that NPY overexpression is inducing BDNF up-regulation through a positive regulation of CREB phosphorylation. However, the mechanisms underlying the increase of BDNF induced by NPY overexpression in MJD mouse models should be further investigated.

Since neuroinflammation was previously implicated in MJD pathology (Evert *et al.*, 2001, Silva-Fernandes *et al.*, 2010, Goncalves *et al.*, 2013), we evaluated the effects of NPY overexpression in some neuroinflammation players. Our results underline that NPY overexpression significantly reduced the mRNA levels of proinflammatory cytokine *Il6* and prevented mutant ataxin-3 induced increase of microglial immunoreactivity. These results are in accordance with former reports describing an anti-inflammatory-like effect of NPY in a microglia cell line, characterized by the inhibition of IL1β increase and nitric oxide production (Ferreira *et al.*, 2010), and reduction of microglia motility, decreasing the local number of activated microglia (Ferreira *et al.*, 2012). Furthermore, BDNF also proved to be able to attenuate neuroinflammation, by reducing astrocytosis, microcytosis and proinflammatory cytokines levels (Bovolenta *et al.*, 2010, Wu *et al.*, 2011). Hence, NPY, directly and/or through the activation of BDNF signaling pathway, restrains the exacerbation of the inflammatory response, preventing the toxic effects of microglia overactivation. This may, at least partially, explain the beneficial effects of NPY overexpression on MJD mouse model.

The present study also showed that NPY overexpression reduced the number of mutant ataxin-3 aggregates without affecting the levels of soluble mutant ataxin-3. This reduction of aggregates accumulation suggests that NPY overexpression may activate protein clearance mechanisms, particularly autophagy, which was previously showed to be impaired both in patients and rodent models of MJD (Nascimento-Ferreira *et al.*, 2011). Considering that it has been recently demonstrated the ability of NPY to induce autophagy (Aveleira *et al.*, 2015), autophagy stimulation may be a NPY-induced mechanism responsible for the reduction of mutant ataxin-3 aggregates. However, further studies are needed to characterize NPY effects over autophagy in MJD mouse models. Additionally, since proinflammatory signals may lead to abnormal processing of proteins promoting their aggregation (Yan *et al.*, 2003, Maccioni *et al.*, 2009), we hypothesize that NPY overexpression may be indirectly decreasing mutant ataxin-3 aggregation as a result of its actions in reduction of proinflammatory signals.

NPY up-regulation mitigates neuroinflammation through the activation of $Y_1$ receptor (Ferreira *et al.*, 2011, Ferreira *et al.*, 2012), and increases autophagy through the activation of $Y_1$ and $Y_5$ receptors (Aveleira *et al.*, 2015), leading to the hypothesis that the NPY neuroprotective effects in these MJD mouse models might be mediated by these two NPY receptors. Further studies using specific NPY receptor knock-outs should be taken to assess the role of each NPY receptor in MJD.

In conclusion, this work provides the first evidence that striatal NPY-positive interneurons are reduced in MJD and that striatal NPY overexpression mitigates the disease-associated neuropathology. Furthermore, our results suggest that increase of BDNF levels and reduction of neuroinflammation are implicated in the beneficial effects of NPY on MJD pathology. Because there is no effective therapy able to stop or prevent the progression of the disease, NPY overexpression might be a novel therapeutic candidate strategy for this disorder.

# Chapter 3

# Cerebellar neuropeptide Y overexpression alleviates motor deficits and neuropathology in a transgenic mouse model of Machado-Joseph disease

## 3.1. Introduction

Machado-Joseph disease (MJD), also known as spinocerebellar ataxia type 3 (SCA3), that was first identified in Portuguese descendants, is currently the most frequent dominantly-inherited ataxia worldwide (Paulson, 2007), particularly in Flores island, Azores, where it reaches the prevalence of 1:239 (Bettencourt *et al.*, 2008). MJD is a polyglutamine (polyQ) disorder because it is caused by an abnormal expansion of CAG trinucleotide repeats within the open reading frame of the *ATXN3* gene, which translates into the protein ataxin-3 with an elongated chain of glutamines: more than 61 in MJD patients (Kawaguchi *et al.*, 1994, Maciel *et al.*, 2001). The expression of mutant ataxin-3 leads to neurodegeneration in specific CNS regions, such as cerebellum, pons, *substantia nigra* and striatum (Sudarsky and Coutinho, 1995, Durr *et al.*, 1996, Klockgether *et al.*, 1998). The clinical hallmarks include progressive ataxia and postural instability, dystonia, ophthalmoplegia, fasciculation-like movements of facial and lingual muscles and bulging eyes (Sudarsky and Coutinho, 1995, Bettencourt and Lima, 2011). There are currently no therapies available to prevent or stop the disease, thus MJD progresses for 1 or 2 decades culminating in patients' death.

Neuropeptide Y (NPY) is a 36-aminoacid peptide abundantly and unequally distributed in several mammalian brain regions. NPY is involved in many physiological functions, through the activation of NPY receptors, $Y_1$, $Y_2$, $Y_4$ and $Y_5$ receptors (Silva *et al.*, 2005), and its levels are altered in some neurodegenerative disorders (Dawbarn *et al.*, 1985, Chan-Palay *et al.*, 1986, Beal *et al.*, 1988a, Beal *et al.*, 1988b, Davies *et al.*, 1990, Mazurek *et al.*, 1997). As NPY proved to be protective towards diverse neurodegenerative conditions, including AD (Croce *et al.*, 2013), PD (Decressac *et al.*, 2012) and HD (Decressac *et al.*, 2010), as well in a striatal mouse model of MJD, we now investigated NPY levels in cerebellum of MJD patients and transgenic mice and the ability of cerebellar NPY overexpression, using a gene therapy approach, to rescue motor and balance impairments displayed by MJD transgenic mice.

Our work shows NPY levels are decreased in MJD cerebella of both patients and transgenic mice and that NPY overexpression mitigates MJD-related motor and balance deficits and the associated cerebellar neuropathology in transgenic mice, suggesting that it may provide a new therapeutic strategy for MJD.

## 3.2. Materials and methods

### 3.2.1. Human brain tissue

Post-mortem human brain tissue from dentate nucleus was obtained from University of Gröningen, The Netherlands. The tissues included a control patient (male, 68 years) and two MJD patients both females: MJD148 with 70 years and 68 CAG repeats, and MJD176 with 34 years and 77 CAG repeats.

### 3.2.2. Animals

C57Bl/6-background transgenic mouse model expressing the C-terminal-truncated ataxin-3 with 69 glutamine repeats and an N-terminal haemagglutinin (HA) epitope driven by Purkinje-cell-specific L7 promoter were obtained from parallel breeding at our research center (CNC) of a colony of transgenic mice initially obtained from Gunma University Graduate School of Medicine (Torashima *et al.*, 2008). Genotyping was performed by PCR. Gender- and age-matched transgenic (Tg) and wild-type (WT) littermates at 5 weeks of age were used in this study.

The experiments were carried out in accordance with the European Community directive (2010/63/EU) covering the protection of animals used for scientific purposes. The researchers received adequate training (FELASA-certified course) and certification to perform the experiments from the Portuguese authorities (Direcção Geral de Alimentação e Veterinária).

### 3.2.3. Viral vectors

AAV serotype 1/2 vectors encoding NPY or EGFP under the control of human synapsin promoter (Sousa-Ferreira *et al.*, 2011) were used.

### 3.2.4. *In vivo* injection of viral vectors into mice cerebellum

After anesthesia of the mice with Avertin (280 µg/g, intraperitoneally), viral vectors were stereotaxically injected into the cerebellum in the following coordinates: anteroposterior -2.3 mm, lateral 0 mm and ventral - 3.0 mm, relative to lambda (Paxinos *et al.*, 2001).

For evaluation of neuroprotection in the MJD transgenic model, Tg and WT littermate animals received a single central 3 µL injection of AAV-1/2-NPY or AAV-1/2-EGFP ($0.67x10^8$ t.u.) in the cerebellum. After the injection, the syringe was left in place for an additional 5 minutes to allow the diffusion of the viral vectors and minimize backflow.

### 3.2.5. Behavioral assessment

Mice were subjected to locomotor tests before and 4 and 8 weeks after viral vectors injection. Animals were habituated for 1h to a quiet room with controlled temperature and ventilation, dimmed lighting, and handled prior to behavioral testing.

*Stationary rotarod*. Motor coordination and balance were assessed using rotarod apparatus (Letica Scientific Instruments, Panlab, Spain), at a constant speed of 5 rpm, over a period of 5 min. The time during which mice remain walking in the rotation drum was recorded. Sessions consisting of two trials per day with a 20-min inter-trial interval were carried out and the mean of the trials was averaged.

*Beam walking*. Motor coordination and balance of mice were assessed by measuring the ability of the mice to traverse a graded series of narrow beams to reach an enclosed safety platform. The beams consisted of long strips of wood (1 m) with an 18- or 9-mm square wide and a 9- or 6-mm round diameter cross-sections, placed horizontally, 25 cm above the bench surface. Mice performed two consecutive trials on each beam, progressing from the widest to the narrowest beam, and the mean latency time to traverse the beam was taken to analysis. Any animal that did not cross within 60 seconds was allocated a maximum value of 60 second for analysis.

*Footprint patterns*. To obtain footprints, the forepaws and hindpaws of the mice were coated with black and white nontoxic paints, respectively. Mice were allowed to walk on a paper along a 100x10x15 cm runway. A sequence of six consecutive steps was chosen for evaluation, excluding footprints made at the beginning and end of the run where the animal was initiating and finishing movement, respectively. Base width was measured as the average distance between left and right footprints. Overlap between forepaw and hindpaw placement is measured as the distance between the front and hind footprints on each side. The mean of the twelve strides and of the six overlap distance for each animal was considered.

### 3.2.6. Immunohistochemical procedure

After an overdose of Avertin (2.5 x 280 µg/g intraperitoneally), transcardial perfusion of the mice was performed with PBS followed by fixation with 4% paraformaldehyde (PFA; Sigma-Aldrich, USA). The brains were removed and post-fixed in 4% PFA for 24h and cryoprotected by incubation in 25% sucrose/PBS for 48h. The brains were frozen and 35 µm sagittal sections were cut using a cryostat (LEICA CM3050S, Germany) at -21ºC. Sections throughout one-half of the cerebellum were collected in anatomical series and stored in 48-well trays as free-floating sections in PBS supplemented with 0.05 µM sodium azide (Sigma-Aldrich), at 4ºC.

Double staining for NPY (rabbit anti-NPY; 1:6000; Sigma-Aldrich), HA (mouse monoclonal anti-HA; 1:1000; InvivoGen, France), Iba1 (rabbit anti-Iba1; 1:1000; Wako, Germany) and nuclear marker [4',6-diamidino-2-phenylindole (DAPI); Sigma-Aldrich] were performed. After RT 1h incubation with blocking solution, and overnight 4ºC incubation with primary antibodies, sections were washed and incubated for 2h at RT with the corresponding secondary antibodies coupled to fluorophores (1:200; Molecular Probes, Life Technologies, USA) diluted in the respective blocking solution. The sections were then mounted in Mowiol reagent (Sigma-Aldrich) on microscope slides.

Staining was visualized using Zeiss Axioskop 2 plus imaging microscope (Carl Zeiss Microimaging, Germany) using 5x and 20x objectives and the AxioVision 4.7 software package (Carl Zeiss Microimaging).

Quantitative analysis was performed with a semiautomated image-analysis software package (ImageJ 1.42q software, USA).

### 3.2.7. Cresyl violet staining

Premounted sagittal sections were stained with cresyl violet (Sigma-Aldrich) for 2 minutes, differentiated in 70% ethanol, dehydrated by passing through 95% ethanol, 100% ethanol and xylene solutions, and mounted onto microscope slides with Eukitt®.

### 3.2.8. Cell counts of mutant ataxin-3 inclusions in Purkinje cells and of Purkinje cells

Sagittal sections were scanned with a 20x objective. All HA aggregates in Purkinje cells, as well as all Purkinje cells, of 8 sections at 280 µm intervals were manually counted and the average number of inclusions and of cells was extrapolated to the whole cerebellum.

### 3.2.9. Evaluation of cerebellar volume

Cerebellar volume was analyzed by photographing, with a 1.25x objective, eight sagittal sections per animal (35 µm thick sections at 280 µm intervals), starting in the middle of the cerebellum by quantifying the area of both cerebellum and cerebrum. The volume was then estimated with the following formula: volume = $d(a_1 + a_2 + a_3 + \ldots)$, where $d$ is the distance between serial sections (200 µm) and $a_1 + a_2 + a_3$ are cerebellar or cerebral areas for individual serial sections (de Almeida *et al.*, 2002). The hemicerebellar and the hemicerebral volume was then multiplied by two to extrapolate the entire cerebellar and cerebral volume.

### 3.2.10. Quantification of granular and molecular layers thickness

Lobules V and IX granular and molecular layers thickness was assessed by the mean of three different measures to each layer, in three sections at 280 µm intervals, scanned with a 20x objective. In each image, boundary lines around the granular and the molecular layers were drawn by hand using ImageJ software.

### 3.2.11. Quantification of Iba1 immunoreactivity

The immunoreactivity indexes were measured through optic density analysis of the affected cerebellar regions relative to their corresponding non-affected cortex (defined as background).

### 3.2.12. Isolation of mRNA and cDNA synthesis

After an overdose of Avertin mice were sacrificed by cervical dislocation and cerebella were dissected and stored in tubes containing RNAlater RNA stabilization reagent (QIAGEN, Germany) at -80°C until extraction of RNA. Total RNA was isolated using the Nucleospin RNA Isolation Kit (Macherey-Nagel, Germany) according to the manufacturer's instructions. Total amount of RNA was quantified using a Nanodrop 2000 Spectrophotometer (Thermo Scientific, USA) and the purity was evaluated by measuring the ratio of OD at 260 and 280 nm. cDNA was then obtained by conversion of 1 µg of total RNA using iScript Selected cDNA Synthesis kit (Bio-Rad) according to the manufacturer's instructions and stored at -20ºC.

### 3.2.13. Quantitative real-time polymerase chain reaction (qRT-PCR)

Quantitative PCR was performed in a StepOnePlus Real-Time PCR system (Applied Biosystems). The primers for the mouse genes – *Npy*, *Hprt* and *Gapdh* – and human genes – *Npy* and *Actin1b* – were pre-designed and validated by QIAGEN (QuantiTect Primers, QIAGEN). A master mix was prepared for each primer set containing the appropriate volume of SsoAdvanced SYBR Green Supermix (Bio-Rad), QuantiTect Primers and template cDNA. All reactions were performed in duplicate and according to the manufacturer's recommendations: 95ºC for 30 sec, followed by 40 cycles at 95ºC for 5 sec and 60ºC for 30 sec. The melting curve protocol started immediately after amplification. The amplification efficiency for each primer pair and the threshold cycle (Ct) were determined automatically by the StepOne Software (Applied Biosystems). The mRNA fold change with respect to control samples was determined by the Pfaffl method, taking into consideration amplification efficiencies of all genes.

### 3.2.14. Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Statistical comparisons were performed using unpaired Student's t-test or one-way or two-way ANOVA followed by Bonferroni post-hoc test. Significance thresholds were set at $p<0.05$, $p<0.01$ or $p<0.001$.

## 3.3. Results

### 3.3.1. NPY levels are decreased in cerebellum of MJD patients and transgenic mice

In order to investigate NPY levels in MJD we analyzed human post-mortem tissue and samples from transgenic mice of the disease.

Dentate nucleus extracts of post-mortem brains of two MJD patients revealed a 92-98% reduction of *Npy* mRNA levels relative to a control sample (Fig. 3.1A). Moreover, cerebellar extracts of 34 week-old MJD Tg animals expressing truncated mutant ataxin-3 with 69 glutamines also displayed a robust significant decrease (*** $p$<0.001, n=3-4) of *Npy* mRNA levels, when compared with WT mice (Fig. 3.1B).

These results show that mutant ataxin-3 expression reduces NPY levels in MJD.

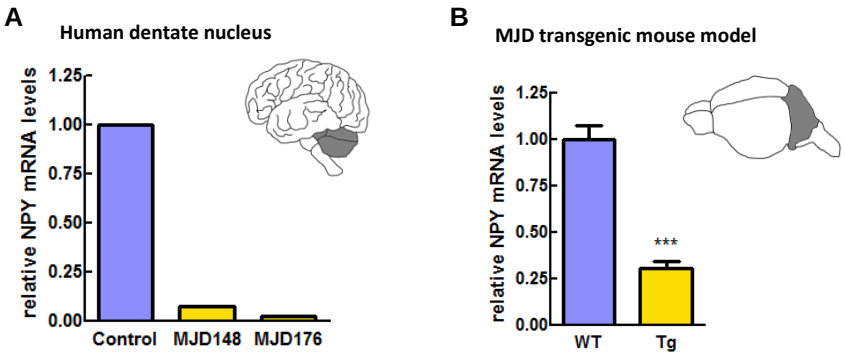


**Figure 3.1 –** NPY levels are reduced in MJD patients and transgenic mice. Quantitative PCR analysis revealed (**A**) a 92 and 98% depletion of *Npy* mRNA levels in extracts obtained from dentate nucleus of two patients with MJD and (**B**) a 60% reduction in extracts derived from dissected cerebella of MJD Tg mouse model (*** $p$<0.001, n=3/4) at 34 weeks of age. Statistical significance was evaluated with unpaired Student's t-test. Data are expressed as mean ± SEM.

### 3.3.2. Experimental strategy used to overexpress NPY in the cerebellum of a transgenic mouse model of MJD.

To evaluate NPY positive effects in MJD-associated motor deficits and cerebellar neuropathology we overexpressed NPY in MJD transgenic mice.

For that purpose, we injected AAV vectors encoding NPY, or EGFP, as control, centrally in the cerebella of 5-week-old Tg mice and WT littermates (Fig. 3.2A). AAV-NPY injection induced a robust NPY expression in cerebellar nuclei and in the layers of the

cerebellar cortex in most lobules, as evidenced by a higher cerebellar NPY-immunoreactivity (Fig. 3.2B and C).

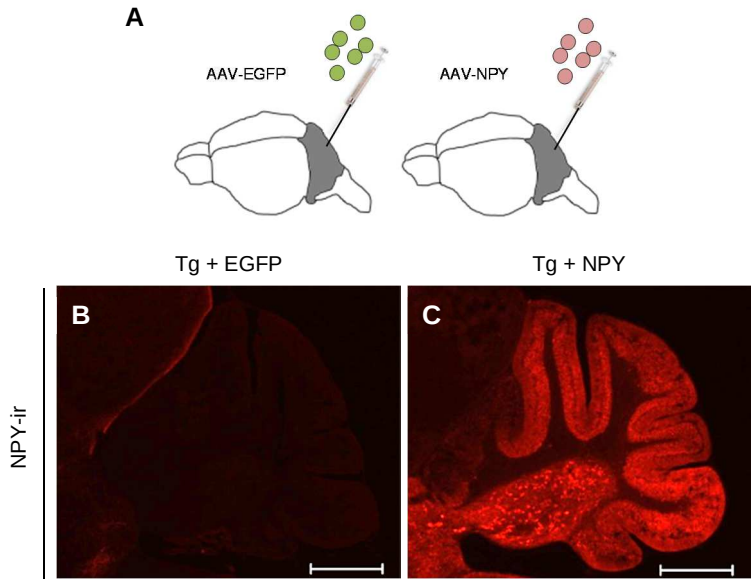


**Figure 3.2 –** AAVs promoted a robust NPY overexpression in mice cerebella. (**A**) Five-week-old MJD Tg and WT littermate mice were centrally injected in the cerebellum with AAV vectors encoding NPY or EGFP, as control. (**B** and **C**) Fluorescence staining for NPY, showing that the stereotaxic injection of the viral vectors allowed the overexpression of NPY. Scale bars: 500 µm.

### 3.3.3. Cerebellar NPY overexpression alleviates balance and motor coordination impairments.

We recently showed that MJD Tg mice display a severe motor coordination impairments (Nascimento-Ferreira *et al.*, 2013). Since NPY levels are reduced in these animals and neuroprotective roles for NPY have been described (Alvaro *et al.*, 2008b, Xapelli *et al.*, 2008, Decressac *et al.*, 2010, Ferreira *et al.*, 2010, Decressac *et al.*, 2012, Ferreira *et al.*, 2012, Croce *et al.*, 2013, Santos-Carvalho *et al.*, 2013), we investigated whether NPY overexpression would improve the MJD phenotype. For that purpose, we performed behavioral tests to assess motor coordination and balance before and 4 and 8 weeks post-injection.

Motor coordination and balance were assessed by the stationary rotarod test. Before stereotaxic surgeries, 5-weeks-old Tg mice displayed a marked phenotype characterized by difficulties walking and equilibrating on the rotating rod when compared with WT littermates, as evidenced by a diminished latency to fall of the rod (Fig. 3.3A). At 8 weeks after vectors

injection, it became clear that cerebellar NPY overexpression significantly prevented this balance and motor coordination impairment (* $p$<0.05 relative to Tg+EGFP, n=8-13).

Moreover, in the beam walking test, Tg animals took more time to cross the beams than WT mice. By 8 weeks post-injection, NPY-overexpressing animals showed a significantly better performance in round beams (Fig. 3.3B; * $p$<0.05 and *** $p$<0.001, n=8-13).

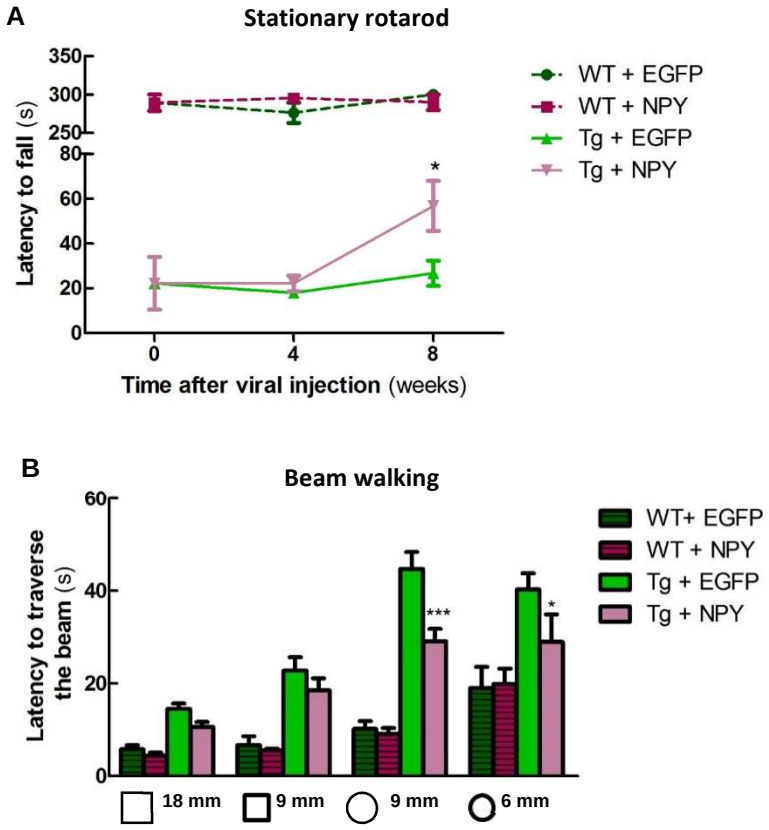


**Figure 3.3 –** NPY overexpression alleviates balance and motor coordination impairments. (**A**) Motor coordination was assessed using stationary rotarod test which showed that Tg animals performed poorly on a stationary rotarod when compared with WT, revealing a diminished latency to fall of the rod, which was significantly prevented (* $p$<0.05 relative to Tg+EGFP, n=8-13) at 8 weeks post-injection by NPY overexpression. (**B**) Balance and motor coordination was measured by the latency time for each animal to traverse a series of progressively more difficult beams of square and round cross-section. At eight weeks after viral vectors injection, Tg mice took more time to cross beams when compared with WT; however, NPY overexpression significantly improved Tg mice performance in the round beams (* p<0.05 and *** p<0.001 relative to Tg+EGFP, n=8-13). Statistical significance was evaluated with two-way ANOVA followed by Bonferroni *post hoc* test. Data are expressed as mean ± SEM.

Taken together these results indicate that NPY overexpression alleviates motor coordination and balance disabilities.

### 3.3.4. NPY overexpression prevents the early development of MJD-like ataxic gait of transgenic mice.

These MJD Tg mice display a severe ataxic gait, as evidenced by a smaller stride length and a widening of the gait base (Nascimento-Ferreira *et al.*, 2013). Thus, to evaluate whether NPY overexpression can impact in ataxic gait, we evaluated Tg mice footprint patterns.

The footprint pattern analysis 4 weeks after stereotaxic injection revealed that Tg mice with cerebellar NPY overexpression showed an almost complete rescue of base width enlargement (Fig. 3.4A, ** $p$<0.01 relative to WT, n=6-8) and footprint overlap distance (Fig. 3,4B, * $p$<0.05 relative to WT and # $p$<0.05 relative to Tg+EGFP, n=6-8).

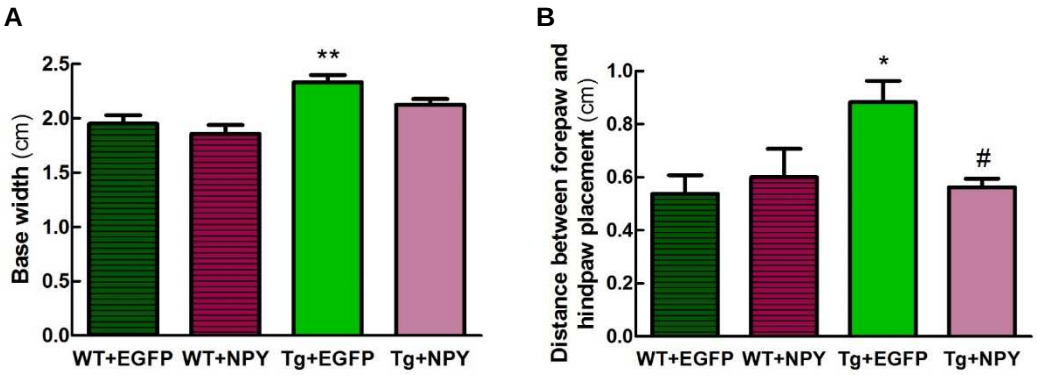


**Figure 3.4 –** NPY overexpression prevents the early development of MJD-like ataxic gait of transgenic mice. The quantitative analysis of footprint patterns 4 weeks post-injection revealed that NPY overexpressing Tg mice present (**A**) an almost complete rescue of base width (** $p$<0.01 relative to WT, n=6-8), and (**B**) an amelioration of footprint overlap (* p<0.05 relative to WT and # p<0.05 relative to Tg+EGFP). Statistical significance was evaluated with one-way ANOVA followed by Bonferroni *post hoc* test. Data are expressed as mean ± SEM.

These data indicate that NPY overexpression prevents MJD-like ataxic gait in transgenic mice.

### 3.3.5. NPY overexpression prevents cerebellar neurodegeneration.

To evaluate whether the phenotypic improvements were due to the prevention of cerebellar neurodegeneration, we compared brains of NPY overexpressing and control Tg mice regarding its size and histopathological parameters, 8 weeks after viral vectors injection.

Cresyl violet-stained cross-sectional areas were used to examine the cerebellar volume relative to the whole brain (Fig. 3.5A and B). Tg mice submitted to cerebellar NPY overexpression showed reduced cerebella volume shrinkage (Fig. 3.5C, * $p$<0.05, n=6-8) compared with Tg+EGFP mice. Furthermore, the analysis of cerebellar layers showed that Tg+NPY mice exhibit a larger granular layer thickness (Fig. 3.5D-F, ** $p$<0.01, n=6-8), compared with control Tg mice.

Overall, these results show that NPY overexpression in the cerebellum of MJD Tg mice preserves cerebellar structure.

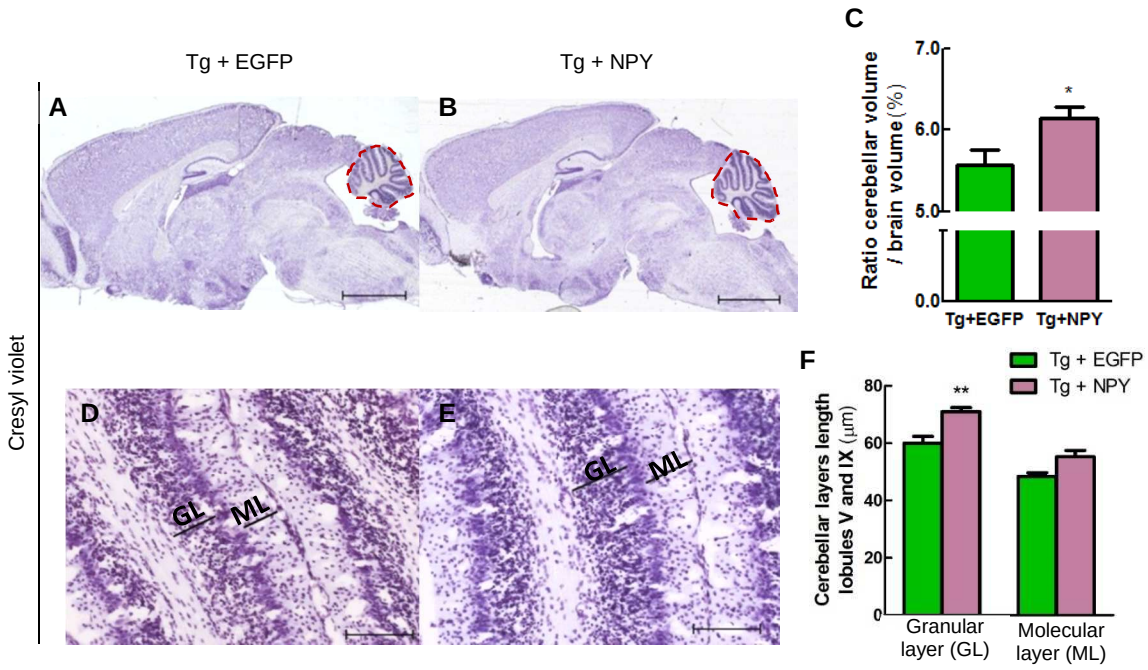


**Figure 3.5 –** NPY overexpression preserves cerebellar structure. (**A**-**B** and **D**-**E**) Midsagittal cresyl violet-stained sections from Tg mice. NPY overexpressing Tg mice (**B**) presented a higher percentage of cerebellar volume relative to cerebral volume (* $p$<0.05, n=6-8) than control EGFP-expressing Tg mice (**A**), as quantified in (**C**). Representative images of cerebellar granular (GL) and molecular (ML) layers from Tg + EGFP (**D**) and Tg + NPY (**E**) animals, confirming the quantification analysis (**F**), which showed that NPY overexpression prevented the reduction of granular layer thickness in lobules V and IX (** $p$<0.01, n=6-8). Statistical significance was evaluated with unpaired Student's t-test. Data are expressed as mean ± SEM. Scale bars: 2000 μm (A and B) and 100 μm (D and E).

### 3.3.6. NPY reduces mutant ataxin-3 aggregates present in Purkinje cells.

We also evaluated NPY overexpression effects in the number of mutant ataxin-3 aggregates in cerebellar Purkinje cells of MJD transgenic mice.

Immunofluorescence labeling of Purkinje cells with an anti-HA antibody, which stains mutant ataxin-3 (Fig. 3.6A and B), showed that NPY overexpression promoted a decrease in the number of mutant ataxin-3 aggregates in Purkinje cells, compared with Tg+EGFP mice (Fig. 3.6C, * $p$<0.05, n=6-8), without decreasing the number of Purkinje cells (Fig, 3.6D).

Altogether, these results show that NPY overexpression decreases the number of mutant ataxin-3 aggregates.

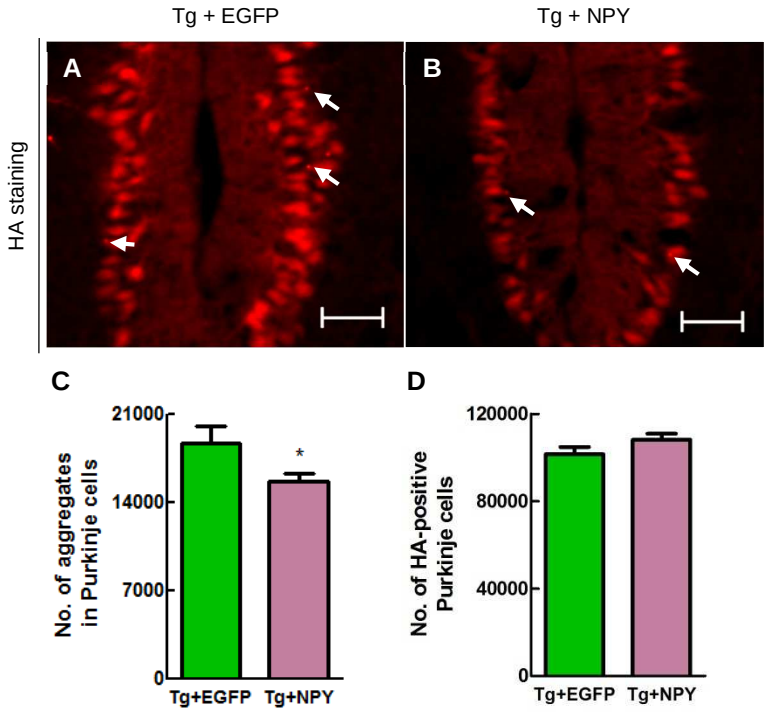


**Figure 3.6 –** NPY overexpression reduces the number of mutant ataxin-3 aggregates present in Purkinje cells. (**A** and **B**) Immunostaining of mutant ataxin-3 with an anti-HA antibody, revealing Purkinje cells. Some Purkinje cells exhibited mutant ataxin-3 aggregates (arrows). As quantified in (**C**), NPY overexpression decreased the number of aggregates in Purkinje cells (* p<0.05, n=6-8), without decreasing the total number of Purkinje cells (**D**, n=6-8). Statistical significance was evaluated with unpaired Student's t-test. Data are expressed as mean ± SEM. Scale bars: 50 µm (A and B).

### 3.3.7. NPY decreases cerebellar neuroinflammation in the transgenic model of MJD.

Considering that neuroinflammation has been argued to be one of the pathogenic mechanisms in MJD (Evert *et al.*, 2001, Silva-Fernandes *et al.*, 2010, Goncalves *et al.*, 2013), we investigated the anti-inflammatory effect of NPY overexpression in MJD transgenic mice.

Strong immunoreactivity for Iba1 was detected in transgenic mice cerebella (Fig. 3.7A and B), revealing microglia activation. Eight weeks post-injection, NPY overexpression induced a decrease of Iba1 immunoreactivity relative to control Atx3MUT+EGFP (Fig. 3.7C, * $p<0.05$, $n=4$).

These results indicate that NPY overexpression attenuate neuroinflammation associated with MJD.

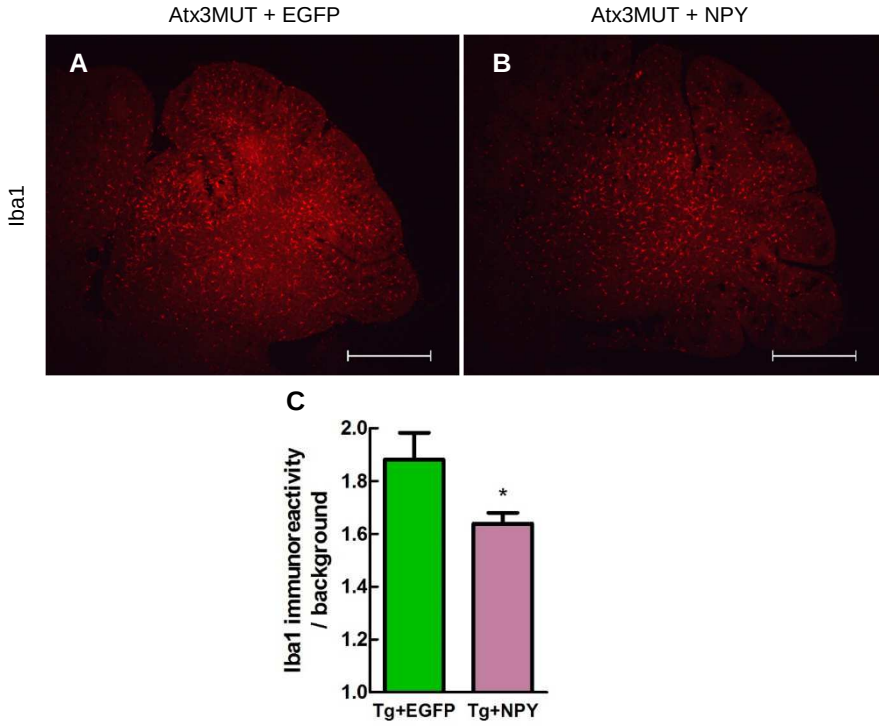


**Figure 3.7 –** NPY overexpression decreases cerebellar neuroinflammation in transgenic model of MJD. (**A** and **B**) Fluorescence staining for ionized calcium-binding adapter molecule 1 (Iba1), a microglia marker. NPY overexpression reduced microglia activation, as evidenced by a decrease of Iba1 immunoreactivity, at 8 weeks after viral injection (* $p<0.05$, $n=6$-8), as quantified in (**C**). Statistical significance was evaluated with unpaired Student's t-test. Data are expressed as mean ± SEM. Scale bars: 500 µm.

## 3.4. Discussion

In this work, we showed that NPY levels are decreased in MJD cerebella of both patients and transgenic mice and that NPY overexpression is able to mitigate MJD-related motor deficits and cerebellar neuropathology in transgenic mice. Since there is no effective therapy able to stop or prevent the progression of the disease, NPY overexpression might be a novel therapeutic candidate strategy for this disorder.

The transgenic MJD mouse model used in this work is characterized by the expression of a truncated form of pathogenically expanded ataxin-3 in the cerebellum (Torashima *et al.*, 2008), which is widely accepted as the most important contributor for the pathology and phenotype presented by MJD patients.

We found that *Npy* levels were reduced in both postmortem cerebellar extracts of MJD patients and transgenic mice. This reduction of NPY levels in MJD is in line with the reduction of striatal NPY-positive interneurons and striatal *Npy* levels, discussed earlier, and with previous reports showing decreased NPY levels in another neurodegenerative disease, namely AD, in both patients (Chan-Palay *et al.*, 1986, Davies *et al.*, 1990) and rodent models (Ramos *et al.*, 2006). Hence, we hypothesized that the described cerebellar neurodegeneration in MJD (Durr *et al.*, 1996, Taniwaki *et al.*, 1997, Klockgether *et al.*, 1998) may also affect the NPY-producing neurons, causing the observed decrease of NPY levels.

We have earlier shown that NPY overexpression through viral gene therapy was able to alleviate MJD neuropathology in a striatal mouse model of MJD, providing proof of principle that NPY may be a potential therapeutic strategy for this disease. Therefore, in this study we investigated whether NPY overexpression in the cerebellum was able to mitigate MJD-associated ataxic phenotypic.

The cerebellum has major importance in motor coordination and balance, amongst other features more recently described (reviewed in (D'Angelo and Casali, 2012)). Indeed, the most characteristic sign of cerebellar damage is ataxia, which consists in movement abnormalities affecting gait, balance, gaze and speech (reviewed in (Ilg and Timmann, 2013)). The transgenic mice used in this study exhibit a pronounced ataxic phenotype as early as 5 weeks of age (Nascimento-Ferreira *et al.*, 2013). In fact, MJD transgenic mice present balance and motor coordination deficits, as assessed by stationary rotarod and beam walking tests, which were rescued by cerebellar NPY overexpression. Furthermore, transgenic mice exhibit ataxic gait, evidenced by an increase of base width and of the distance between hindpaw and forepaw placement, features that were completely rescued by cerebellar NPY overexpression. The clinical manifestation of MJD starts with gait imbalance and progresses to wide-base gait ataxia and limb incoordination (Paulson, 2007), thus NPY overexpression may be a promising strategy to overcome these motor

impairments. However, further studies would be needed to disclose the efficacy and safety of this therapy in humans.

Moreover, the alleviation of behavioral defects correlated with a reduction of cerebellar disease-related neuropathology, namely a preservation of the cerebellar volume and of the granular layer thickness.

The observed beneficial effects on motor function of NPY is in accordance with previous reports in HD transgenic mice where an ICV injection of NPY reduced their behavioral disabilities, as evidenced by improving rotarod performance and reducing paw-clasping (Decressac *et al.*, 2010). These effects were also related with a preservation of striatal integrity (Decressac *et al.*, 2010).

The present study also showed that NPY overexpression reduced the number of mutant ataxin-3 aggregates in Purkinje cells without affecting the total number of these cerebellar cells. As discussed earlier, this reduction of aggregates accumulation suggests that NPY overexpression may activate protein clearance mechanisms, particularly autophagy, which is impaired both in patients and rodent models of MJD (Nascimento-Ferreira *et al.*, 2011). Although it has been recently demonstrated the ability of NPY to induce autophagy (Aveleira *et al.*, 2015), further studies are needed to characterize NPY effects over autophagy in MJD mouse models. Furthermore, considering that proinflammatory signals may lead to abnormal processing of proteins promoting their aggregation (Yan *et al.*, 2003, Maccioni *et al.*, 2009), we can suggest that NPY overexpression may be indirectly decreasing mutant ataxin-3 aggregation as a result of its actions in reduction of proinflammatory signals.

In fact, NPY-mediated anti-inflammatory effects have been observed in in microglia cell lines (Ferreira *et al.*, 2010, Ferreira *et al.*, 2012) and also in the MJD lentiviral-based striatal model, as observed earlier. In this study, we observed that cerebellar NPY overexpression reduced mutant ataxin-3-induced increase of microglia activation, as evidenced by a decrease in immunoreactivity of a microglial marker. Furthermore, considering that neuroinflammation was previously implicated in MJD pathology (Evert *et al.*, 2001, Silva-Fernandes *et al.*, 2010, Goncalves *et al.*, 2013), the NPY-mediated reduction of microglial activation, and the consequent prevention of toxic effects of microglia overactivation, may, at least in part, be responsible for the neuroprotective effects of NPY on MJD transgenic mouse model.

Considering that this transgenic mice expresses only a C-terminal fragment of mutant ataxin-3, this model may also represent a more general polyQ disease model. Thus, we can hypothesize that NPY overexpression may be neuroprotective in other polyQ diseases, and ataxias. This hypothesis is further supported by previous observations of NPY positive effects in HD (Decressac *et al.*, 2010).

In conclusion, this work provides the first evidence that NPY levels are reduced in the cerebellum of MJD patients and of transgenic mice and that cerebellar NPY overexpression alleviates motor- and balance-related deficits, as well as cerebellar neuropathology. Furthermore, we reinforce the observation of attenuation of neuroinflammation as a potential NPY-mediated neuroprotective mechanism on MJD pathology. This supports NPY overexpression or administration as a candidate strategy to modulate the abnormal neuropathological and motor changes in MJD.

# CHAPTER 4

# Hypothalamic changes in a transgenic mouse model of Machado-Joseph disease

## 4.1. Introduction

Machado-Joseph disease (MJD), also called spinocerebellar ataxia type 3 (SCA3), is an autosomal dominant neurodegenerative disease caused by an over-repetition of the trinucleotide CAG in the *ATXN3* gene (Kawaguchi *et al.*, 1994). This genetic mutation translates into an expanded polyglutamine (polyQ) stretch that confers a toxic gain-of-function to the ataxin-3 (Atx3) protein, leading to neuronal dysfunction and cell death (Paulson *et al.*, 1997b). The most affected brain regions are cerebellum, pons, substantia nigra and striatum (Sudarsky and Coutinho, 1995, Durr *et al.*, 1996, Klockgether *et al.*, 1998), which are responsible for the hallmark symptoms of the disease: progressive ataxia, dysfunction of the motor coordination and balance that affect gait, speech and gaze (Paulson, 2007). However, MJD patients also present non-motor symptoms, like sleep disturbances (Schols *et al.*, 1998, Pedroso *et al.*, 2011, dos Santos *et al.*, 2014), weight loss (Riess *et al.*, 2008, Saute *et al.*, 2012), depression (Cecchin *et al.*, 2007) and deterioration of memory and learning abilities (Roeske *et al.*, 2013).

Hypothalamus is a CNS region involved in the regulation of energy homeostasis, sleep and emotion (reviewed in (Swaab, 2004)). It consists on a number of anatomically well-organized and interconnected nuclei. Each of these nuclei comprises neuronal populations expressing specific neuropeptides, namely the *arcuate nucleus* (Arc) contains Neuropeptide Y (NPY) / Agouti-related protein (AgRP)- and pro-opiomelanocortin (POMC) / cocaine- and amphetamine-regulated transcript (CART)-expressing neurons and the lateral hypothalamus (LH) contains orexin (Orx)- and melanin-concentrating hormone (MCH)-expressing neurons. The hypothalamic dysfunction and degeneration has already been implicated in neurodegenerative disorders, such as AD (Callen *et al.*, 2001, Loskutova *et al.*, 2010), PD (Politis *et al.*, 2008b) and HD (Kassubek *et al.*, 2004, Douaud *et al.*, 2006, Politis *et al.*, 2008a, Soneson *et al.*, 2010, Politis *et al.*, 2011, Gabery *et al.*, 2015), and this neuropathology was already associated with some symptoms of these diseases (Raadsheer *et al.*, 1995, Petersen *et al.*, 2005, Hult *et al.*, 2011, Hult Lundh *et al.*, 2013).

Considering that MJD patients also present some non-motor symptoms that may be associated with hypothalamic alterations, we hypothesized the involvement of hypothalamus in MJD neuropathology. Thus, in this study, we investigated if hypothalamic neuronal populations associated with sleep and energy homeostasis regulation are altered in two MJD transgenic mouse models: CAMKII Tg mice that express a full length expanded ataxin-3 in the forebrain (Mayford *et al.*, 1996, Boy *et al.*, 2009) and L7 Tg mice which express a truncated form of the mutant atx3 in the cerebellar Purkinje cells (Torashima *et al.*, 2008).

Our results showed a reduction of Orx-, MCH- and POMC-positive hypothalamic neurons of CAMKII MJD Tg mice, and no changes were observed regarding hypothalamic NPY levels. Moreover, this hypothalamic neuropathology was possibly due to mutant ataxin-

3 accumulation in this brain region. These hypothalamic changes of MJD may be accountable for some non-motor symptoms of MJD patients. Thus, for the first time, we identified hypothalamus as a new therapeutic target in MJD.

## 4.2. Materials and methods

### 4.2.1. MJD transgenic mice

A transgenic mouse model was generated by crossbreeding the promoter line expressing the tetracycline transactivator (tTA) combined with calcium/calmodulin dependent kinase II (CamKII) promoter, to achieve both regional and temporal control of the transgene expression (Mayford *et al.*, 1996), with the stable responder mouse line number 2904, containing the full-length human ataxin-3c isoform (GenBank accession number: U64820), which has 77 CAG repeats (Boy *et al.*, 2009). Ten-month-old transgenic (Tg) mice and wild-type (WT) littermates were obtained from University of Tübingen, Germany, courtesy of Dr. Thorsten Schmidt.

C57Bl/6-background transgenic mouse model expressing the C-terminal-truncated ataxin-3 with 69 glutamine repeats and an N-terminal haemagglutinin (HA) epitope driven by Purkinje-cell-specific L7 promoter were obtained from parallel breeding at our research center (CNC) of a colony of transgenic mice initially obtained from Gunma University Graduate School of Medicine (Torashima *et al.*, 2008). Genotyping was performed by PCR. Gender- and age-matched Tg and WT littermates at 7 months of age were used in this study.

The experiments were carried out in accordance with the European Community directive (2010/63/EU) covering the protection of animals used for scientific purposes. The researchers received adequate training (FELASA-certified course) and certification to perform the experiments from the Portuguese authorities (Direcção Geral de Alimentação e Veterinária).

### 4.2.2. Immunohistochemical procedure

After an overdose of Avertin (2.5 x 280 µg/g intraperitoneally), transcardial perfusion of the mice was performed with PBS followed by fixation with 4% paraformaldehyde (PFA; Sigma-Aldrich, USA). The brains were removed and post-fixed in 4% PFA for 24h and cryoprotected by incubation in 25% sucrose/PBS for 48 h. The brains were frozen and 25 µm coronal sections were cut using a cryostat (LEICA CM3050S, Germany) at -21ºC. Sections throughout the entire hypothalamus were collected in anatomical series and stored in 48-well trays as free-floating sections in PBS supplemented with 0.05 µM sodium azide (Sigma-Aldrich), at 4ºC.

The immunohistochemical procedure was initiated by endogenous peroxidase quenching with 30 min incubation at 37ºC in 0.1% phenylhydrazine (Merck, USA) /PBS

solution. Sections were then kept at room temperature (RT) for 1 h in PBS with 0.1% Triton X-100 (Sigma-Aldrich) containing 10% goat serum (NGS; Gibco, Alfagene, Portugal), then overnight at 4ºC in blocking solution with the primary antibodies: a rabbit polyclonal anti-orexin-A (OrxA) antibody (1:1000; Phoenix-Pharmaceuticals, USA) or a rabbit polyclonal anti-POMC antibody (1:500; Phoenix-Pharmaceuticals). After washing, the sections were incubated with the respective biotinylated secondary antibodies (1:200; Vector Laboratories, USA). Bound antibodies were visualized using the VECTASTAIN® ABC kit, with 3,3'-diaminobenzidine tetrahydrochloride (DAB metal concentrate; Pierce, USA) as substrate. The sections were mounted, dehydrated and coverslipped with Eukitt® (Sigma-Aldrich).

Double staining for MCH (rabbit anti-MCH; 1:200; Phoenix-Pharmaceuticals), OrxA (rabbit anti-OrxA; 1:1000; Phoenix-Pharmaceuticals), POMC (rabbit anti-POMC; 1:500; Phoenix-Pharmaceuticals), mutant ataxin-3 (mouse anti-1H9; 1:5000; Chemicon, Merck-Millipore, USA), HA (mouse anti-haemagglutinin; 1:1000; InvivoGen, France), NPY (rabbit anti-NPY; 1:6000; Sigma-Aldrich) and nuclear marker [4',6-diamidino-2-phenylindole (DAPI); Sigma-Aldrich] were performed. After RT 1 h incubation with blocking solution, and overnight 4ºC incubation with primary antibodies, sections were washed and incubated for 2 h at RT with the corresponding secondary antibodies coupled to fluorophores (1:200; Molecular Probes, Life Technologies, USA) diluted in the respective blocking solution. The sections were then mounted in Mowiol reagent (Sigma-Aldrich) on microscope slides.

Staining was visualized using Zeiss Axioskop 2 plus imaging microscope (Carl Zeiss Microimaging, Germany) using 5x and 20x objectives and the AxioVision 4.7 software package (Carl Zeiss Microimaging). Co-localizations were visualized using a confocal LSM 510 Meta microscope (Carl Zeiss Microimaging) using 40x and 63x objectives.

Quantitative analysis was performed with a semiautomated image-analysis software package (ImageJ 1.42q software, USA).

### 4.2.3. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

After immunohistochemistry, brain sections were washed twice and then incubated for 1h30 at 37ºC with the TUNEL mix (in situ cell death kit; Roche Applied Science, Mannheim, Germany) and washed again. Coverslips were mounted in Mowiol mounting media and images were acquired using a confocal LSM 510 Meta microscope (Carl Zeiss Microimaging) using 40x and 63x objectives.

### 4.2.4. Cell counts of Orx-, MCH- and POMC-positive neurons

Ten coronal sections at 200 µm intervals that encompassed the entire lateral hypothalamic (LH) area were scanned with a 20x objective. All Orx- and MCH-positive neurons were manually counted, and its number extrapolated for the entire LH region.

Eight coronal sections at 200 µm intervals that encompassed the entire arcuate nucleus (Arc) were scanned with a 20x objective. All POMC-positive neurons were manually counted, and its number extrapolated for the entire Arc.

### 4.2.5. Quantification of cross-sectional soma length of Orx-, MCH- and POMC-positive neurons

The biggest cross-sectional soma length of Orx-, MCH- and POMC-positive neurons was measured in 15 randomly selected positive neurons per section, in a total of 6 sections per animal, using ImageJ software. The mean value was calculated.

### 4.2.6. Quantification of NPY immunoreactivity

Eight coronal sections at 200 µm intervals that encompassed the entire Arc were simultaneously subjected to immunohistochemistry procedure and to photographs acquisition, with the same exposure time. A constant area that includes the Arc was used to measure the immunoreactivity indexes through optic density analysis in each hemisphere. These measurements were relative to a corresponding non-NPY-positive hypothalamic region in the same brain section (defined as background).

### 4.2.7. Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Statistical comparisons were performed using unpaired Student's t-test. Significance thresholds were set at $p<0.05$, $p<0.01$ or $p<0.001$.

## 4.3. Results

### 4.3.1. Orx-positive neurons are reduced in the hypothalamus of CAMKII MJD transgenic mice

Considering that Orx is expressed by a large neuronal population in the LH and it is associated with sleep regulation and feeding behavior (Sakurai *et al.*, 1998, Chemelli *et al.*, 1999, Thannickal *et al.*, 2000, Hara *et al.*, 2001), we investigated the number of Orx-positive neurons in a transgenic mouse model of the disease, CAMKII MJD mice.

Hypothalamic Orx neurons were stained in brain sections of Tg mice expressing mutant ataxin-3 under the control of CAMKII promoter (Fig. 4.1B and C) and of WT littermates (Fig. 4.1A and C). CAMKII Tg mice present a 29% reduction in the number of Orx-positive neurons relative to WT mice (Fig. 4.1E, * $p$<0.05, n=6). Moreover, no differences were found in the cross-sectional soma length of these neurons in the two experimental groups (Fig. 4.1F, ns $p$>0.05, n=6), and thus no evidence point to a morphometric alteration of the remaining Orx neurons in transgenic hypothalamus.

These results show that CAMKII Tg mice have reduced hypothalamic Orx neurons.

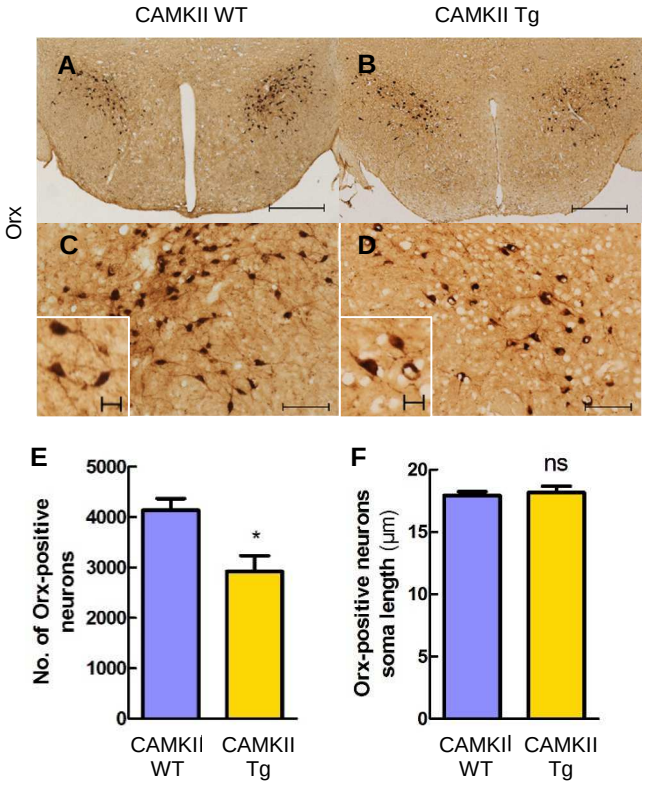


**Figure 4.1 –** CAMKII MJD transgenic mice show loss of hypothalamic Orx-positive neurons. (**A-D**) Immunohistochemical peroxidase staining using an anti-OrxA antibody. CAMKII Tg mice (**B**

and **D**) have reduced number of Orx neurons when compared with WT littermates (**A** and **C**), as quantified in **E** (* p<0.05, n=6). There are no differences on the biggest soma cross-sectional length of these neurons between the two experimental groups (**F**, ns p>0.05, n=6). Statistical significance was evaluated with unpaired Student's t-test. Data are expressed as mean ± SEM. Scale bars: 500 µm (A and B), 100 µm (C and D) and 20 µm (C and D, higher magnifications).

### 4.3.2. MCH-positive hypothalamic neurons are decreased in CAMKII MJD transgenic mice

MCH-expressing neurons are also an important and expressive neuronal population in the LH and have been implicated in sleep and energy homeostasis regulation (Qu *et al.*, 1996, Shimada *et al.*, 1998, Verret *et al.*, 2003, Willie *et al.*, 2008). Thus, we investigated the number and size of these neurons in the CAMKII MJD Tg mouse model.

Immunofluorescence of hypothalamic MCH neurons (Fig. 4.2A-D) revealed that the number of these neurons are decreased in CAMKII MJD transgenic mice when compared to WT littermates (Fig. 4.2E, * *p*<0.05, n=6). Additionally, there are no differences on the cross-sectional length of these neurons in both animal groups (Fig. 4.2F, ns *p*>0.05, n=6).

Altogether, these results show that MCH-positive neurons are reduced in MJD Tg mice, although the surviving MCH neurons are not morphologically altered.

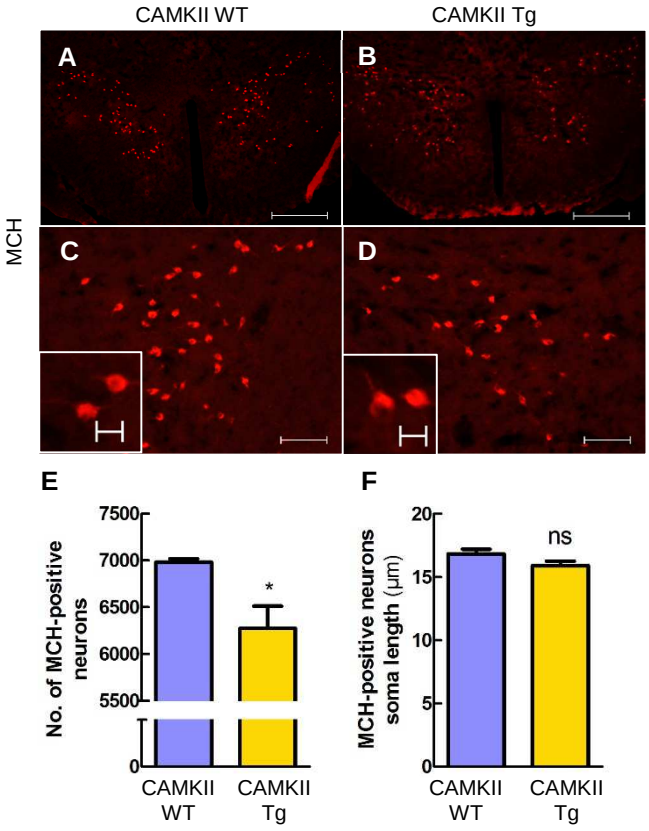

**Figure 4.2 –** CAMKII MJD transgenic mice present loss of hypothalamic MCH-positive neurons. (**A**-**D**) Fluorescence staining for MCH. Hypothalamus of CAMKII Tg mice (**B** and **D**) had less MCH neurons when compared with WT littermates (**A** and **C**), as quantified in **E** (* p<0.05, n=6). There are no differences on the biggest soma cross-sectional length of these neurons between the two experimental groups (**F**, ns p>0.05, n=6). Statistical significance was evaluated with unpaired Student's t-test. Data are expressed as mean ± SEM. Scale bars: 500 µm (A and B), 100 µm (C and D) and 20 µm (C and D, higher magnifications).

### 4.3.3. CAMKII MJD transgenic mice hypothalamus have reduced number of POMC-positive neurons

The Arc is a hypothalamic nucleus with important roles in energy homeostasis and one of its predominant neuronal populations is constituted by neurons expressing POMC, which are anorexigenic neurons since they inhibit food intake (Valassi *et al.*, 2008).

To assess whether POMC-positive neurons are altered in MJD mice hypothalamus, we stained and counted POMC neurons in brain sections of Tg (Fig. 4.3B) and WT (Fig. 4.3A) mice. We observed that CAMKII MJD Tg mice present a reduction of POMC-positive neurons relative to WT littermates (Fig. 4.3C; 2979 ± 322 versus 4163 ± 210 control, * $p$<0.05, n=6). Furthermore, there are no significant differences in the size of the remaining POMC-positive neurons in the Tg hypothalamus relative to the WT (Fig 4.3D, ns $p$>0.05, n=6), as assessed by measuring the cross-sectional surface length of the cell bodies.

These results suggest that POMC-positive neurons are decreased in MJD mice and there are no changes in the size of the surviving POMC neurons.

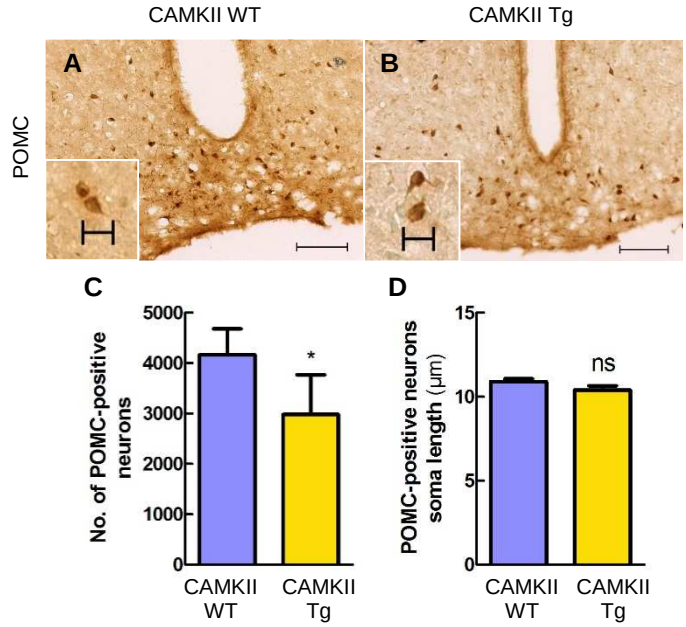

**Figure 4.3 –** CAMKII MJD transgenic mice revealed loss of hypothalamic POMC-positive neurons. (**A** and **B**) Immunohistochemical peroxidase staining using an anti-POMC antibody. Hypothalamus of CAMKII Tg mice (**B**) had reduced number of POMC-immunopositive neurons when compared with WT littermates (**A**), as quantified in **C** (* p<0.05, n=6), although their size do not differ between animals (**D**, ns p>0.05, n=6). Statistical significance was evaluated with unpaired Student's t-test. Data are expressed as mean ± SEM. Scale bars: 500 µm and 20 µm (A and B, higher magnifications).

### 4.3.4. Hypothalamic NPY levels are unchanged in CAMKII MJD transgenic mice

Considering that NPY is very abundant in the hypothalamus, particularly in Arc, and its levels are altered in the most affected brain areas of neurodegenerative diseases, such as MJD (as observed in chapters 2 and 3), we assessed NPY immunoreactivity (ir) in the Arc of CAMKII MJD mice.

There were no differences in NPY-ir between CAMKII MJD Tg (Fig. 4.6B and D) and WT (Fig. 4.6A and C) mice (Fig. 4.6E; ns *p*>0.05, n=6), which suggest that NPY levels might be preserved in the mutant ataxin-3-expressing hypothalamus.

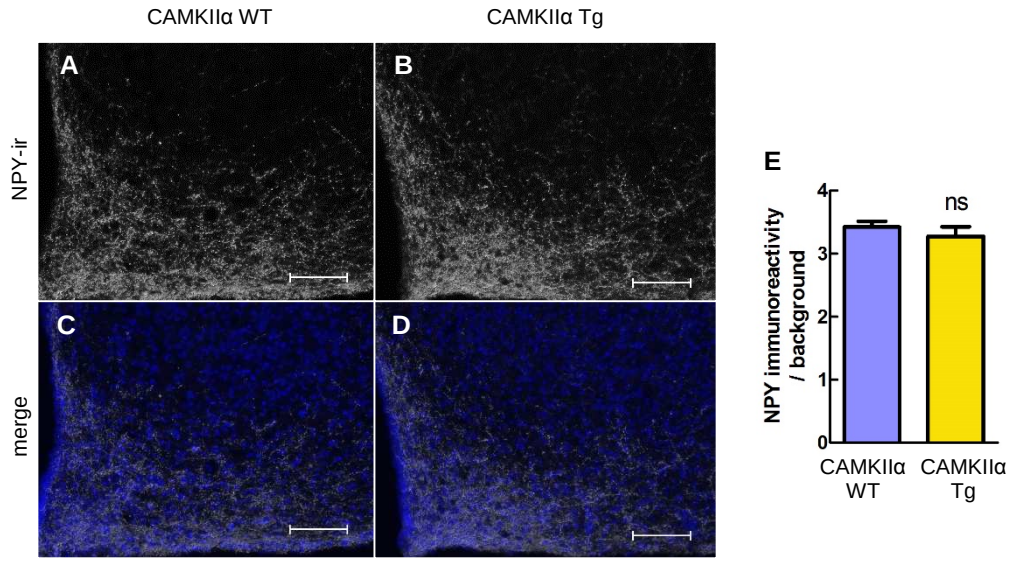


**Figure 4.4 –** No changes of NPY immunoreactivity in CAMKII MJD transgenic and WT mice. (**A**-**D**) Fluorescence staining for NPY (white) and cells nuclei (blue). Bilateral quantification of NPY immunoreactivity (ir) displayed no differences between WT (**A**) and Tg (**B**) mice (**E**, ns p>0.05, n=6). Statistical significance was evaluated with unpaired student's t-test. Data are expressed as mean ± SEM. Scale bars: 100 µm.

### 4.3.5. Mutant ataxin-3 is accumulated in CAMKII MJD mice hypothalamus possibly leading to neuronal death

In order to get better insight into how MJD may be affecting hypothalamic neuronal populations, we investigated the presence of mutant ataxin-3 in these neurons.

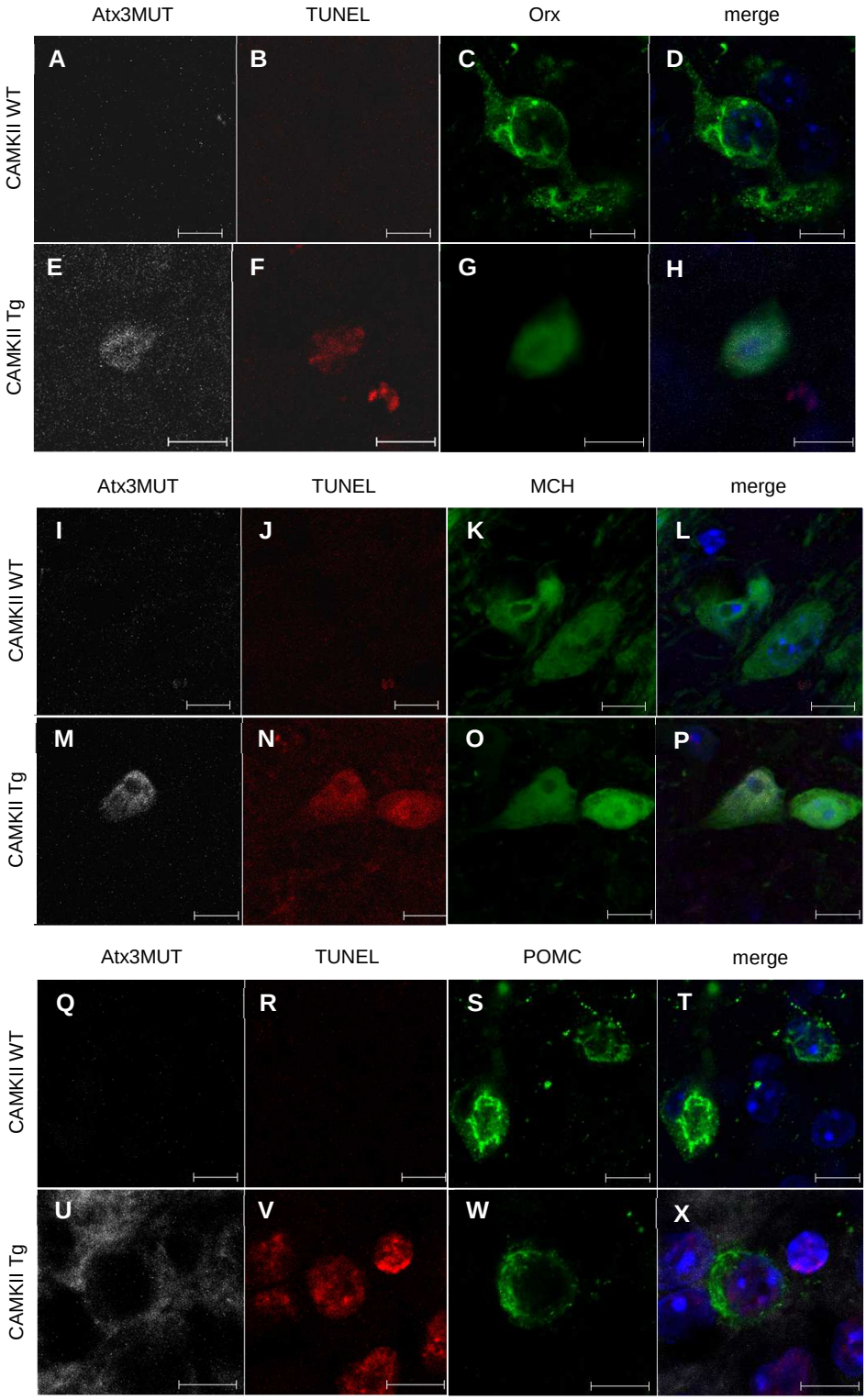

**Figure 4.5 –** Mutant ataxin-3 is present in neurons of CAMKII MJD transgenic mice hypothalamus, possibly leading to apoptosis in Orx-, MCH- and POMC-positive neurons. (**A**-**X**) Fluorescence staining for Atx3MUT (1H9 antibody, white), DNA fragmentation (TUNEL assay, red) and Orx, MCH or POMC (green), analysed by laser confocal microscopy analysis. Hypothalamic cells of WT mice do not present mutant ataxin-3 (**A**, **I** and **Q**) nor signs of apoptosis (**B**, **J** and **R**). In CAMKII Tg mice, mutant ataxin-3 is diffusely expressed in the nuclei of some Orx- (**E** and **H**) and MCH-positive cells (**M** and **P**), and in the cytoplasma of POMC-positive cells (**U** and **X**). Most of these cells containing mutant ataxin-3 present signs of apoptosis (**F**, **N** and **V**), suggesting that the expression of mutant ataxin-3 may be leading to cell death in these neuronal populations of the hypothalamus. Scale bar 10 μm.

In WT animals, hypothalamic cells did not express mutant ataxin-3 (Fig. 4.4A, I and Q) and do not show signs of apoptosis (Fig. 4.4B, J and R), as assessed by TUNEL straining. On the other hand, we observed that some Orx- and MCH-positive cells present nuclear mutant ataxin-3 (Fig. 4.4E and H and Fig. 4.4M and P, respectively), while in POMC-positive hypothalamic neurons mutant ataxin-3 is present in the cytoplasm (Fig. 4.4U and X). Furthermore, some of these mutant ataxin-3-expressing neurons show signs of apoptosis (Fig. 4.4F, N and V), suggesting that the mutated protein is leading to cell death in these neuronal populations.

### 4.3.6. L7 MJD transgenic mice, without mutant ataxin-3 expression in the hypothalamus, present no changes in hypothalamic neuronal populations

To support the hypothesis that changes in hypothalamic neuronal populations are due to mutant ataxin-3 expression in the hypothalamus, we investigated possible alterations in Orx-, MCH- and POMC-expressing neuronal populations in the hypothalamic of L7 MJD Tg mice, which express mutant ataxin-3 particularly in cerebellar Purkinje cells (Torashima *et al.*, 2008).

Indeed, L7 MJD Tg mice do not express mutant ataxin-3 in the hypothalamus (Fig. 4.5C and D), namely in LH and Arc, as observed by a lack of immunofluorescence for the HA tag, present in the transgene.

Furthermore, L7 Tg mice do not present differences in the number and size of Orx- (Fig. 4.5E and F; ns $p>0.05$, n=5-6) MCH- (Fig. 4.5G and H; ns $p>0.05$, n=5-6) and POMC-positive neurons (Fig. 4.5I and J; ns $p>0.05$, n=5-6), relative to WT littermates.

Altogether, these data suggest that the absence of mutant ataxin-3 expression in Tg mice hypothalamus may be associated with the lack of differences in the number and size of neurons from the analyzed hypothalamic populations.

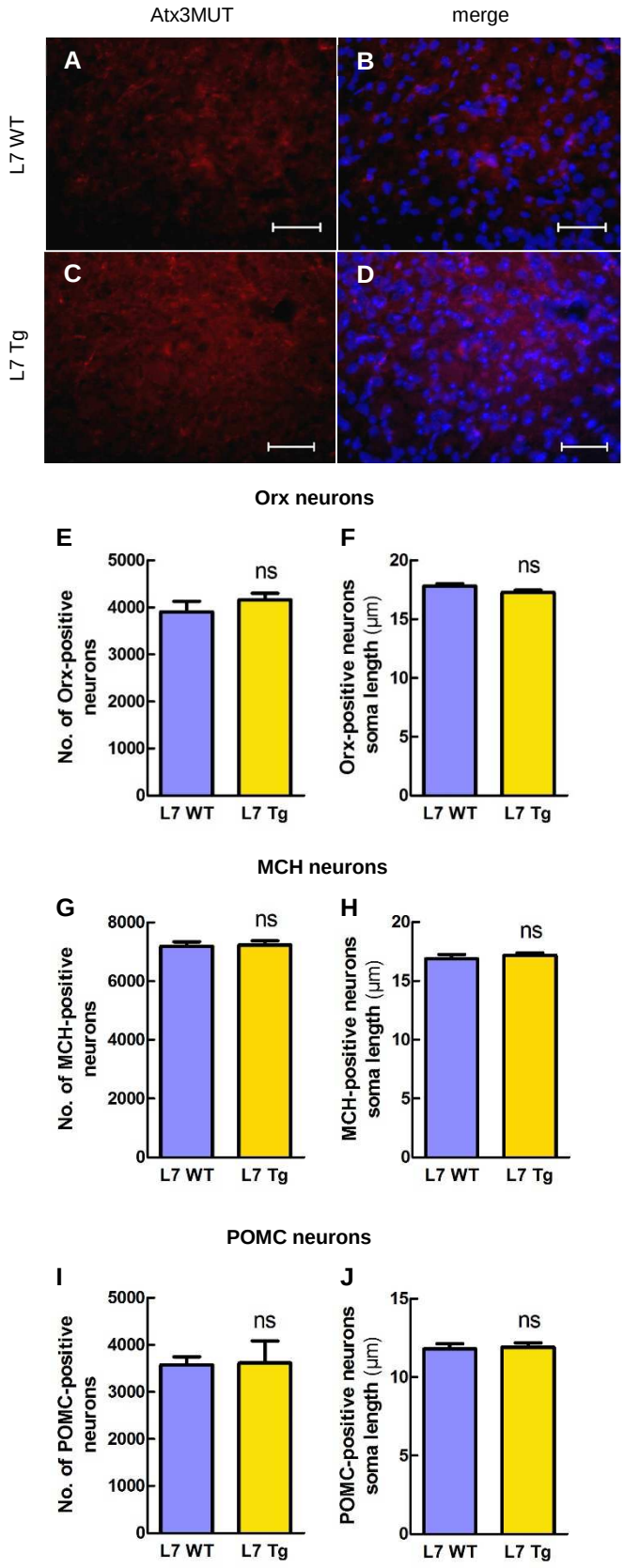


**Figure 4.6 –** Mutant ataxin-3 is not present in L7 MJD transgenic mice hypothalamus and there are no changes in Orx, MCH nor POMC neuronal populations. (**A**-**D**) Fluorescence staining for Atx3MUT (1H9 antibody, red) and cells nuclei (blue). Atx3MUT is not present neither in L7 WT

(**A**) nor in L7 Tg (**C**) mice hypothalamus. L7 Tg mice present no differences in the number of Orx- (**E**), MCH- (**G**) and POMC-positive neurons (**I**) compared with WT littermates (ns $p>0.05$, n=5-6). Similarly, there are no differences on the soma cross-sectional length of these neurons between the two experimental groups (**F**, **H** and **J**, ns $p>0.05$, n=6). Statistical significance was evaluated with unpaired Student's t-test. Data are expressed as mean ± SEM. Scale bar 50 μm.

## 4.4. Discussion

In this study, we showed, for the first time, that hypothalamic changes occur in a MJD transgenic mouse model, consisting in a reduction of Orx-, MCH- and POMC-positive hypothalamic neurons, although no changes were observed regarding hypothalamic NPY levels. The hypothalamic neuropathology was likely due to mutant ataxin-3 accumulation in this brain region.

The hypothalamus is a brain region involved in the regulation of energy homeostasis, sleep and emotion (reviewed in (Swaab, 2004)), and it has already been implicated in neurodegenerative diseases, such as AD (Callen *et al.*, 2001, Loskutova *et al.*, 2010), PD (Politis *et al.*, 2008b) and HD (Kassubek *et al.*, 2004, Douaud *et al.*, 2006, Politis *et al.*, 2008a, Soneson *et al.*, 2010, Politis *et al.*, 2011, Gabery *et al.*, 2015). In fact, the reported reduction of hypothalamic volume and hypothalamic dysfunction can explain, at least partially, some symptoms, like sleep disturbances, weight loss and depression, which are common to most of patients suffering from these disorders.

Orx has been mostly associated with sleep regulation. Orx knock-out (Chemelli *et al.*, 1999) and Orx neuron-ablated mice (Hara *et al.*, 2001) show phenotypes similar to human narcolepsy, and human narcoleptic patients present low CSF levels of Orx (Nishino *et al.*, 2000, Mignot *et al.*, 2002, Knudsen *et al.*, 2010), consistent with a drastic reduction in the number of Orx-positive neurons assessed in postmortem tissue (Peyron *et al.*, 2000, Thannickal *et al.*, 2000). While Orx cells promote wakefulness, MCH cells, the other neuronal population in LH, may promote sleep. Accordingly, MCH knock-out mice spent more time awake (Willie *et al.*, 2008) and ICV injections of MCH increase sleep (Verret *et al.*, 2003). Therefore, the bidirectional communication between Orx and MCH neurons (Burt *et al.*, 2011) is essential for sleep regulation. Our results show that the number of Orx- and MCH-positive neurons are decreased in CAMKII MJD Tg mice hypothalamus. This is in accordance with previous reports in other neurodegenerative disorders, namely AD (Fronczek *et al.*, 2012, Kasanuki *et al.*, 2014), PD (Thannickal *et al.*, 2007) and HD (Petersen *et al.*, 2005, Gabery *et al.*, 2010, Williams *et al.*, 2011). Therefore, although it would be important to confirm a consequent reduction of Orx and MCH levels in the CAMKII MJD Tg mice, we hypothesize that these hypothalamic alterations may be accountable for sleep disturbances reported in MJD patients (Schols *et al.*, 1998, Pedroso *et al.*, 2011, dos Santos *et al.*, 2014).

Moreover, these LH neuronal populations are also involved in food consumption and body weight. It has been shown that ICV injections of either Orx or MCH in rodents lead to increased food intake (Qu *et al.*, 1996, Sakurai *et al.*, 1998, Edwards *et al.*, 1999, Haynes *et al.*, 2000) and both Orx- and MCH-deficient mice are hypophagic (Shimada *et al.*, 1998, Hara *et al.*, 2001). Apart from LH neurons, Arc neurons have key roles in the regulation of energy homeostasis, through its 2 principal neuronal populations: POMC- / CART-expressing

neurons (anorexigenic, since they inhibit food intake) and NPY- / AgRP-expressing neurons (orexigenic, since they stimulate food intake) (reviewed in (Barsh and Schwartz, 2002)). Here, we report a decrease of POMC-positive neurons in CAMKII MJD Tg mice hypothalamus. Although no differences were observed in NPY immunoreactivity levels and further studies are needed to assess each neuropeptide and their receptors levels, alterations in POMC-, Orx- and MCH-expressing neurons may be sufficient to cause the decrease of body weight that is usually reported by MJD patients (Riess *et al.*, 2008, Saute *et al.*, 2012).

Furthermore, the majority of MJD patients also report depressive symptoms (Cecchin *et al.*, 2007). The etiology of depression, especially depression in neurodegenerative disorders patients, is not completely understood, but it has been suggested that it is related with deregulation of hypothalamic-pituitary-adrenal (HPA) axis (Davis *et al.*, 1986, Masugi *et al.*, 1989, Gurevich *et al.*, 1990, Heuser *et al.*, 1991, Leblhuber *et al.*, 1995, Charlett *et al.*, 1998, Aziz *et al.*, 2009, van Duijn *et al.*, 2010, Shirbin *et al.*, 2013). Indeed in AD and HD abnormalities in hypothalamic PVN (the nucleus involved in HPA axis) were reported (Raadsheer *et al.*, 1995, Gabery *et al.*, 2010). However, such PVN analysis have not yet been performed in MJD samples.

In this work we studied two different MJD transgenic mouse models, which, due to differences in the transgene and in the promoter that control its expression, present distinct ages at onset of the disease, different degrees of symptoms severity and different patterns of neuropathology. The CAMKII MJD Tg mice express the full length human mutant Atx3 in the forebrain and present a mild phenotype that takes place several months after birth (Mayford *et al.*, 1996, Boy *et al.*, 2009), while the L7 MJD Tg mice express a truncated form of the mutated Atx3 only in the cerebellar Purkinje cells and thus revealed a severe ataxic phenotype as early as 3 weeks of age (Torashima *et al.*, 2008, Nascimento-Ferreira *et al.*, 2013). Considering that MJD manifests usually in middle life and, although some brain regions are more vulnerable than others, mutant Atx3 is expressed throughout the brain (Paulson *et al.*, 1997b, Schmidt *et al.*, 1998), CAMKII MJD model may be more suited to study hypothalamic neuropathology in this disorder.

To clarify the involvement of mutant Atx3 in the observed hypothalamic changes in the CAMKII MJD mouse model, we investigated the presence of this mutated protein in the diminished neuronal populations. Our results show that in this mouse model mutant Atx3 is expressed in the surviving Orx-, MCH- and POMC-neurons, which also present signs of apoptosis. These hypothalamic alterations were not observed in the L7 MJD mouse model, which do not express mutant Atx3 in this brain region. Hence, we hypothesize that mutant Atx3 accumulation in hypothalamus is responsible for the observed neuropathology. In fact, the mutant Atx3 ability to cause hypothalamic neuronal death was previously demonstrated by Hara *et al.*, who developed a narcolepsy transgenic mouse model in which Orx-containing

neurons are ablated by expression of Atx3 with an expanded polyQ stretch (Hara *et al.*, 2001). However, further studies are needed to evaluate the accumulation of mutant Atx3 in MJD patients' hypothalami and its consequent toxicity.

Atx3 is normally a predominantly cytoplasmic protein, but the expansion of the polyQ tract causes the accumulation of the mutated protein in the nucleus of neurons (Paulson *et al.*, 1997a, Schmidt *et al.*, 1998). The importance of nuclear localization was emphasized by Yang and colleagues, who showed that polyQ aggregates localized to the cytoplasm had little or no cytotoxicity, but lead to dramatic cell death when localized to nuclei (Yang *et al.*, 2002). Furthermore, nuclear localization accelerated and intensified the motor impairments of MJD transgenic mice (Bichelmeier *et al.*, 2007). Nevertheless, there are some conflicting studies, which suggest that the presence of the expanded protein in the nucleus is not sufficient to cause neurodegeneration nor the development of disease symptoms (Trottier *et al.*, 1998, Warrick *et al.*, 1998, Boy *et al.*, 2010, Silva-Fernandes *et al.*, 2010). In this study, we observed an accumulation of mutant atx3 in the nucleus of Orx- and MCH-positive neurons and in the cytoplasm of POMC-neurons. Although further studies will be needed to disclose the importance of nuclear localization of mutant Atx3 in the hypothalamic neurons, we observed a reduced number of Orx- and MCH-positive neurons, as well as of POMC-expressing neurons, suggesting that both nuclear as cytoplasmic localization of mutant Atx3 affect MJD hypothalamus.

Considering Arc NPY-immunoreactivity, NPY levels are not affected in CAMKII MJD Tg mice hypothalami. However, it is possible that the number of NPY-positive neurons are equally decreased but the content of NPY per cell is increased resulting in unchanged levels.

In the present study, to the best of our knowledge, for the first time, we provide evidence that the hypothalamus is a CNS regions that is also affected by MJD neuropathology, since we show a reduction in the number of some hypothalamic neuronal populations, associated with mutant Atx3-induced neuronal apoptosis. Despite further studies regarding MJD mice phenotype are still lacking, we suggest that the observed hypothalamic changes are responsible for sleep disturbances and body weight loss reported by MJD patients. This way, we identified hypothalamus as a new therapeutic target in MJD.

# CHAPTER 5

# Final conclusions and future perspectives

## 5. Final conclusions and future perspectives

This thesis provides, for the first time, evidence that NPY levels are reduced in MJD patients and mouse models, and that NPY overexpression in these mouse models mediated alleviation of MJD-associated motor deficits and neuropathology. Moreover, our results show alterations in the hypothalamus of MJD transgenic mice, indicating the hypothalamus as a new therapeutic target of the disease.

NPY levels are reduced in the cerebellar samples of MJD patients and also in cerebellum and striatum of mouse models of the disease, which suggest that mutant Atx3-induced neurodegeneration is also affecting NPY-expressing neurons. This decrease of NPY levels is in line with studies in AD patients' brains (Chan-Palay *et al.*, 1986, Davies *et al.*, 1990, Ramos *et al.*, 2006). However, in HD, striatal NPY-positive interneurons appeared to be preserved (Dawbarn *et al.*, 1985, Beal *et al.*, 1986a, Boegman *et al.*, 1987) and NPY levels were actually increased (Beal *et al.*, 1988b, Mazurek *et al.*, 1997). The reason for this discrepant findings in these neurodegenerative diseases is still unknown, but it may be due to differences in the predominant pathogenic mechanism occurring in each disorder. For instance, even though NPY-positive neurons express the antioxidant Cu/Zn SOD and present low abundance of NMDA receptors, which may confer more resistance to excitotoxic stimulus (Kumar, 2004), cell's response to a specific mutated protein may be relatively constant between different neuronal populations. Hence, we can hypothesize that mutant ataxin-3 neuronal processing, its cleavage and aggregation, may be affecting equally both NPY-positive and NPY-negative striatal neuronal populations.

The exact mechanisms leading to MJD pathogenesis are not fully known, but it has been shown that excitotoxicity (Chen *et al.*, 2008, Koch *et al.*, 2011, Goncalves *et al.*, 2013), neuroinflammation (Evert *et al.*, 2001, Silva-Fernandes *et al.*, 2010, Goncalves *et al.*, 2013), autophagy impairment (Nascimento-Ferreira *et al.*, 2011, Nascimento-Ferreira *et al.*, 2013), proteolysis (Simoes *et al.*, 2012, Simoes *et al.*, 2014) and transcriptional deregulation (Chou *et al.*, 2008) contribute for MJD neurodegeneration. Considering that NPY has previously been shown to exhibit neuroprotective properties, due to its ability: a) to reduce excitotoxicity (Corvino *et al.*, 2012, Santos-Carvalho *et al.*, 2013), b) to decrease neuroinflammation (Ferreira *et al.*, 2011, Ferreira *et al.*, 2012), c) to increase trophic support (Croce *et al.*, 2013) and d) to stimulate autophagy (Aveleira *et al.*, 2015), we hypothesized that NPY could alleviate MJD. Accordingly, our results show that NPY overexpression mediated by AAV vectors in MJD mouse models, i) reduced mutant Atx3 aggregates (chapters 2 and 3), ii) decreased striatal (chapter 2) and iii) cerebellar (chapter 3) neurodegeneration and iv) alleviated balance and motor impairments of transgenic mice (chapter 3). This NPY-induced

neuroprotection is in accordance with previous observations in other neurodegenerative disorders, namely in HD, where ICV NPY injection in transgenic mice reduced the striatal atrophy (Decressac *et al.*, 2010), and in PD, in which a striatal NPY injection preserved the nigrostriatal dopaminergic pathway (Decressac *et al.*, 2012). Furthermore, we observed that NPY overexpression in MJD mice v) increases BDNF levels (chapter 2) and vi) reduces neuroinflammation (chapter 2 and 3). Hence, our work suggest that these mechanisms are implicated in the beneficial effects of NPY on MJD pathology, although we cannot exclude other neuroprotective pathways.

Our results support the use of NPY overexpression as a therapeutic strategy for MJD, which is particularly relevant given that no therapies to stop or delay disease progression are presently available.

Stable overexpression of NPY in the brain using AAV vectors was already demonstrated in rodent models (Noe *et al.*, 2010, Sousa-Ferreira *et al.*, 2011), representing a way to permanently provide a therapeutic protein within the CNS. In the case of neurodegenerative diseases, which are prolonged conditions, a continuous and permanent expression of the therapeutic protein is an advantage when compared to frequent drug administrations. Moreover, clinical trials have been evaluating the therapeutic efficiency and safety of viral-mediated expression of some peptides in individuals suffering from neurodegenerative diseases, such as PD (Christine *et al.*, 2009, Bartus *et al.*, 2013). Nevertheless, due to safety and regulatory restrains to the use of gene therapy as a common clinical practice, an alternative strategy would be the delivery of NPY to the CNS. Several studies in rodents use the ICV injection or the injection in specific brain regions to deliver the NPY to the CNS (Decressac *et al.*, 2010, Decressac *et al.*, 2011, Corvino *et al.*, 2012, Decressac *et al.*, 2012). Since this is an invasive procedure, NPY delivery through the systemic route may be a more adequate clinical approach. In fact, it was already shown that NPY readily enters the brain crossing the blood-brain barrier after intravenous injection (Kastin and Akerstrom, 1999). The intranasal administration route, which constitutes a more direct route towards the brain, reducing the potentially undesirable distribution of the injected NPY to several body organs, was already successfully used to deliver NPY to rodents (Serova *et al.*, 2013, Laukova *et al.*, 2014). Additionally, one clinical trial has already tested this route of NPY administration in healthy humans (NCT00748956) and it is on-going another clinical trial for its use in individuals suffering from post-traumatic stress disorder (NCT01533519), with the aim of investigating the safety of this approach. Nevertheless, future studies are needed to assess if the intranasal administration of NPY could effectively reach MJD most affected brain regions, such as striatum and cerebellum.

To achieve a more specific effect it is important to investigate which NPY receptor or receptors are involved in NPY-induced neuroprotection in MJD. This way, through stereotaxic injection of viral vectors encoding NPY receptor(s) or using selective NPY receptors peptide

agonists, we can assess the potential neuroprotection in MJD mouse models. Alternatively, we can co-inject with the AAV-NPY, viral vectors encoding RNA short-hairpins (shRNA) to silence each NPY receptor, and evaluate which silenced NPY receptor would abrogate the NPY neuroprotective effects. Afterwards, we could test if the co-injection of viral vectors encoding NPY and vectors encoding for the NPY receptor(s) that mediate neuroprotection, would potentially escalate the protective effects. However, one has to take into consideration that compensatory mechanisms might take place when a specific NPY receptor is activated or antagonized. In fact, knock-out mice of the $Y_1$ receptor (Pedrazzini *et al.*, 1998, Kanatani *et al.*, 2000), the $Y_2$ receptor (Sainsbury *et al.*, 2002a), the $Y_4$ receptor (Sainsbury et al., 2002b) and the $Y_5$ receptor (Kanatani et al., 2000) are viable and largely normal.

The hypothalamus is a CNS region involved in the regulation of energy homeostasis, sleep and emotion (reviewed in (Swaab, 2004)). Changes in hypothalamic functions and overall hypothalamic neurodegeneration were identified in AD, PD and HD patients (Callen *et al.*, 2001, Kassubek *et al.*, 2004, Douaud *et al.*, 2006, Politis *et al.*, 2008a, Politis *et al.*, 2008b, Loskutova *et al.*, 2010, Soneson *et al.*, 2010, Politis *et al.*, 2011, Gabery *et al.*, 2015). Additionally, in recent years it has been hypothesised that changes in hypothalamic neuropeptides levels and neuronal death in this brain region are accountable for some non-motor symptoms presented by PD and HD patients (Petersen *et al.*, 2005, Hult *et al.*, 2011, Hult Lundh *et al.*, 2013). To the best of our knoweledge, such evaluation of hypothalamic pathogenesis has not been performed in MJD patients and animal models. In this work, our results showed a reduction of Orx-, MCH- and POMC-positive hypothalamic neurons of CAMKII MJD Tg mice (chapter 4). Moreover, we hypothesize that this hypothalamic neuropathology was due to mutant ataxin-3 accumulation in this brain region, considering that hypothalamic changes were not observed in MJD transgenic mice that did not express the mutated protein in hypothalamic neurons (chapter 4). This is in line with reports in the other neurodegenerative disorders, like AD, PD and HD, in which neurofibrillary tangles and amyloid β plaques, Lewy bodies and mutant huntingtin aggregates, respectively, have been observed in the hypothalamic neurons (Langston and Forno, 1978, Standaert *et al.*, 1991, Gabery *et al.*, 2010).

We suggest that the hypothalamus is a new therapeutic target in MJD based in MJD transgenic mouse models. However, further studies are needed to assess hypothalamic neurodegeneration in MJD patients, taking advantage of cerebral scanning techniques and analysing post-mortem tissue samples. Furthermore, future studies should also be performed to investigate the presence of non-motor symptoms, such as body weight, food intake, sleep patterns and depression, in CAMKII MJD transgenic mice, in an attempt to evaluate a possible causal relationship between hypothalamic pathogenesis and symptomatology.

In conclusion, we have shown that NPY levels are reduced in the cerebellum of MJD patients and in striatum and cerebellum of two MJD mouse models. Moreover, NPY overexpression mediated by stereotaxic injection of AAV vectors into the striatum and cerebellum of those MJD mice reduced neurodegeneration, decreased the number of mutant ataxin-3 aggregates and alleviated balance and motor impairments, suggesting that NPY overexpression can be an effective therapeutic strategy for MJD. Furthermore, our work identifies hypothalamus as a new therapeutic target in MJD, since our results showed a reduction in the number of some neuronal populations in the hypothalamus of MJD transgenic mice.

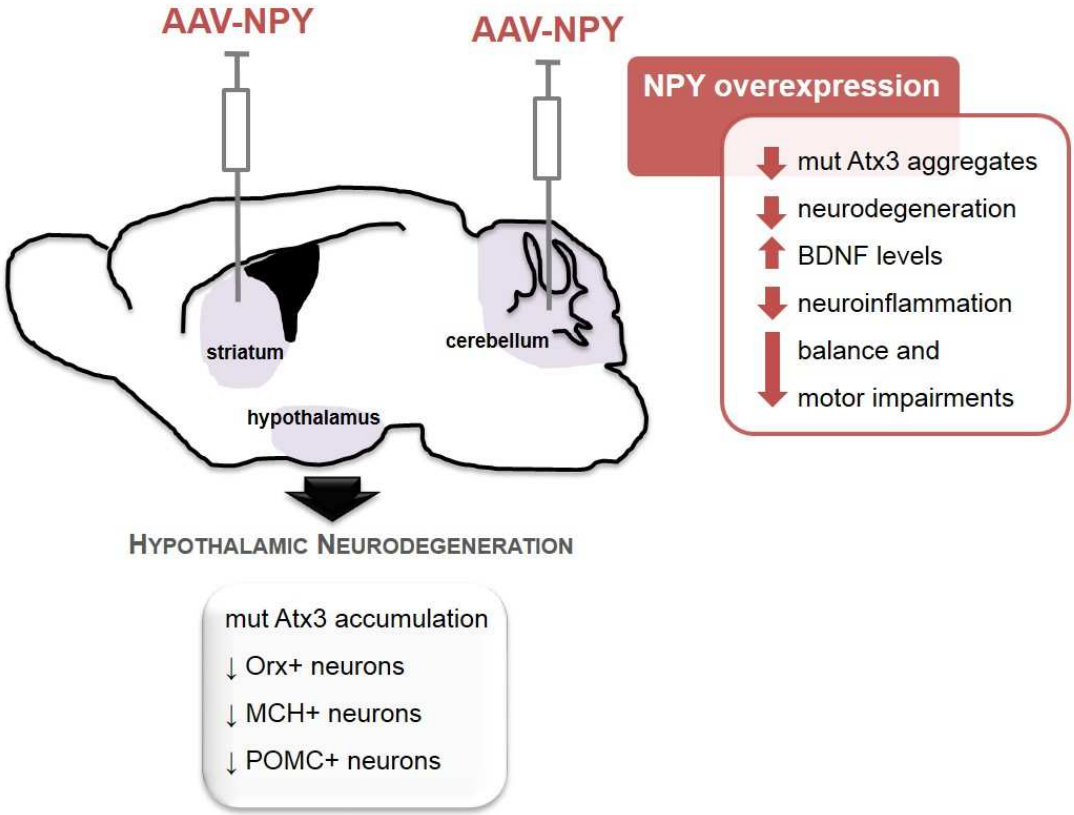


**Figure 5.1 – NPY overexpression for neuroprotection in striatal and cerebellar mouse models of MJD and disease-associated hypothalamic neurodegeneration.** NPY overexpression mediated by AAV vectors in the striatum of a lentiviral-based MJD mouse model and in the cerebellum of transgenic MJD mice reduced mutant Atx3 aggregates, decreased striatal and cerebellar neurodegeneration and alleviated balance and motor impairments of transgenic mice. Additionally, NPY overexpression increased BDNF levels and reduced neuroinflammation, which suggest that these mechanisms are implicated in the beneficial effects of NPY on MJD pathology. Moreover, we observed a reduction of Orx-, MCH- and POMC-positive hypothalamic neurons of MJD transgenic mice and an accumulation of mutant Atx3 in these neuronal populations, indicating the hypothalamus as a new therapeutic target of the disease.

# REFERENCES

**References**

Adachi H, Waza M, Tokui K, Katsuno M, Minamiyama M, Tanaka F, et al. CHIP overexpression reduces mutant androgen receptor protein and ameliorates phenotypes of the spinal and bulbar muscular atrophy transgenic mouse model. J Neurosci. 2007;27(19):5115-26.

Adrian TE, Allen JM, Bloom SR, Ghatei MA, Rossor MN, Roberts GW, et al. Neuropeptide Y distribution in human brain. Nature. 1983;306(5943):584-6.

Agasse F, Bernardino L, Kristiansen H, Christiansen SH, Ferreira R, Silva B, et al. Neuropeptide Y promotes neurogenesis in murine subventricular zone. Stem Cells. 2008;26(6):1636-45.

Al-Ramahi I, Lam YC, Chen HK, de Gouyon B, Zhang M, Perez AM, et al. CHIP protects from the neurotoxicity of expanded and wild-type ataxin-1 and promotes their ubiquitination and degradation. J Biol Chem. 2006;281(36):26714-24.

Albrecht M, Golatta M, Wullner U, Lengauer T. Structural and functional analysis of ataxin-2 and ataxin-3. Eur J Biochem. 2004;271(15):3155-70.

Allen Institute for Brain Science. Allen Mouse Brain Connectivity Atlas. [Internet] http://mouse.brain-map.org/gene/show/73806. 2014 [cited; Available from: http://mouse.brain-map.org/gene/show/73806

Allen JM, Cross AJ, Crow TJ, Javoy-Agid F, Agid Y, Bloom SR. Dissociation of neuropeptide Y and somatostatin in Parkinson's disease. Brain Res. 1985;337(1):197-200.

Allen YS, Adrian TE, Allen JM, Tatemoto K, Crow TJ, Bloom SR, et al. Neuropeptide Y distribution in the rat brain. Science. 1983;221(4613):877-9.

Almeida CG, Takahashi RH, Gouras GK. Beta-amyloid accumulation impairs multivesicular body sorting by inhibiting the ubiquitin-proteasome system. J Neurosci. 2006;26(16):4277-88.

Alvaro AR, Martins J, Araujo IM, Rosmaninho-Salgado J, Ambrosio AF, Cavadas C. Neuropeptide Y stimulates retinal neural cell proliferation--involvement of nitric oxide. J Neurochem. 2008a;105(6):2501-10.

Alvaro AR, Martins J, Costa AC, Fernandes E, Carvalho F, Ambrosio AF, et al. Neuropeptide Y protects retinal neural cells against cell death induced by ecstasy. Neuroscience. 2008b;152(1):97-105.

Alvaro AR, Rosmaninho-Salgado J, Santiago AR, Martins J, Aveleira C, Santos PF, et al. NPY in rat retina is present in neurons, in endothelial cells and also in microglial and Muller cells. Neurochem Int. 2007;50(5):757-63.

Alves S, Nascimento-Ferreira I, Auregan G, Hassig R, Dufour N, Brouillet E, et al. Allele-specific RNA silencing of mutant ataxin-3 mediates neuroprotection in a rat model of Machado-Joseph disease. PLoS One. 2008a;3(10):e3341.

Alves S, Nascimento-Ferreira I, Dufour N, Hassig R, Auregan G, Nobrega C, et al. Silencing ataxin-3 mitigates degeneration in a rat model of Machado-Joseph disease: no role for wild-type ataxin-3? Hum Mol Genet. 2010;19(12):2380-94.

Alves S, Regulier E, Nascimento-Ferreira I, Hassig R, Dufour N, Koeppen A, et al. Striatal and nigral pathology in a lentiviral rat model of Machado-Joseph disease. Hum Mol Genet. 2008b;17(14):2071-83.

Ansari MA, Scheff SW. Oxidative stress in the progression of Alzheimer disease in the frontal cortex. J Neuropathol Exp Neurol. 2010;69(2):155-67.

Antonijevic IA, Murck H, Bohlhalter S, Frieboes RM, Holsboer F, Steiger A. Neuropeptide Y promotes sleep and inhibits ACTH and cortisol release in young men. Neuropharmacology. 2000;39(8):1474-81.

Aoki C, Pickel VM. Neuropeptide Y in the cerebral cortex and the caudate-putamen nuclei: ultrastructural basis for interactions with GABAergic and non-GABAergic neurons. J Neurosci. 1989;9(12):4333-54.

Araujo J, Breuer P, Dieringer S, Krauss S, Dorn S, Zimmermann K, et al. FOXO4-dependent upregulation of superoxide dismutase-2 in response to oxidative stress is impaired in spinocerebellar ataxia type 3. Hum Mol Genet. 2011;20(15):2928-41.

Arvidsson A, Collin T, Kirik D, Kokaia Z, Lindvall O. Neuronal replacement from endogenous precursors in the adult brain after stroke. Nat Med. 2002;8(9):963-70.

Atack JR, Beal MF, May C, Kaye JA, Mazurek MF, Kay AD, et al. Cerebrospinal fluid somatostatin and neuropeptide Y. Concentrations in aging and in dementia of the Alzheimer type with and without extrapyramidal signs. Arch Neurol. 1988;45(3):269-74.

Aveleira CA, Botelho M, Carmo-Silva S, Pascoal JF, Ferreira-Marques M, Nobrega C, et al. Neuropeptide Y stimulates autophagy in hypothalamic neurons. Proc Natl Acad Sci U S A. 2015;112(13):E1642-51.

Aziz NA, Pijl H, Frolich M, van der Graaf AW, Roelfsema F, Roos RA. Increased hypothalamic-pituitary-adrenal axis activity in Huntington's disease. J Clin Endocrinol Metab. 2009;94(4):1223-8.

Aziz NA, van der Marck MA, Pijl H, Olde Rikkert MG, Bloem BR, Roos RA. Weight loss in neurodegenerative disorders. J Neurol. 2008;255(12):1872-80.

Bachoud-Levi AC, Gaura V, Brugieres P, Lefaucheur JP, Boisse MF, Maison P, et al. Effect of fetal neural transplants in patients with Huntington's disease 6 years after surgery: a long-term follow-up study. Lancet Neurol. 2006;5(4):303-9.

Bamford NS, Robinson S, Palmiter RD, Joyce JA, Moore C, Meshul CK. Dopamine modulates release from corticostriatal terminals. J Neurosci. 2004;24(43):9541-52.

Baptista S, Bento AR, Goncalves J, Bernardino L, Summavielle T, Lobo A, et al. Neuropeptide Y promotes neurogenesis and protection against methamphetamine-

induced toxicity in mouse dentate gyrus-derived neurosphere cultures. Neuropharmacology. 2012;62(7):2413-23.

Baraban SC, Hollopeter G, Erickson JC, Schwartzkroin PA, Palmiter RD. Knock-out mice reveal a critical antiepileptic role for neuropeptide Y. J Neurosci. 1997;17(23):8927-36.

Barnea A, Roberts J. Induction of functional and morphological expression of neuropeptide Y (NPY) in cortical cultures by brain-derived neurotrophic factor (BDNF): evidence for a requirement for extracellular-regulated kinase (ERK)-dependent and ERK-independent mechanisms. Brain Res. 2001;919(1):57-69.

Barnea A, Roberts J, Keller P, Word RA. Interleukin-1beta induces expression of neuropeptide Y in primary astrocyte cultures in a cytokine-specific manner: induction in human but not rat astrocytes. Brain Res. 2001;896(1-2):137-45.

Barsh GS, Schwartz MW. Genetic approaches to studying energy balance: perception and integration. Nat Rev Genet. 2002;3(8):589-600.

Bartus RT, Baumann TL, Siffert J, Herzog CD, Alterman R, Boulis N, et al. Safety/feasibility of targeting the substantia nigra with AAV2-neurturin in Parkinson patients. Neurology. 2013;80(18):1698-701.

Bauer PO, Nukina N. The pathogenic mechanisms of polyglutamine diseases and current therapeutic strategies. J Neurochem. 2009;110(6):1737-65.

Baydyuk MaX, B. BDNF in Huntington's Disease: Role in Pathogenesis and Treatment, Huntington's Disease - Core Concepts and Current Advances. Dr Nagehan Ersoy Tunali (Ed). 2012.

Beal MF, Clevens RA, Mazurek MF. Somatostatin and neuropeptide Y immunoreactivity in Parkinson's disease dementia with Alzheimer's changes. Synapse. 1988a;2(4):463-7.

Beal MF, Kowall NW, Ellison DW, Mazurek MF, Swartz KJ, Martin JB. Replication of the neurochemical characteristics of Huntington's disease by quinolinic acid. Nature. 1986a;321(6066):168-71.

Beal MF, Mazurek MF, Chattha GK, Svendsen CN, Bird ED, Martin JB. Neuropeptide Y immunoreactivity is reduced in cerebral cortex in Alzheimer's disease. Ann Neurol. 1986b;20(3):282-8.

Beal MF, Mazurek MF, Ellison DW, Swartz KJ, McGarvey U, Bird ED, et al. Somatostatin and neuropeptide Y concentrations in pathologically graded cases of Huntington's disease. Ann Neurol. 1988b;23(6):562-9.

Beauchemin AM, Gottlieb B, Beitel LK, Elhaji YA, Pinsky L, Trifiro MA. Cytochrome c oxidase subunit Vb interacts with human androgen receptor: a potential mechanism for neurotoxicity in spinobulbar muscular atrophy. Brain Res Bull. 2001;56(3-4):285-97.

Bedard A, Gravel C, Parent A. Chemical characterization of newly generated neurons in the striatum of adult primates. Exp Brain Res. 2006;170(4):501-12.

Bedford L, Hay D, Devoy A, Paine S, Powe DG, Seth R, et al. Depletion of 26S proteasomes in mouse brain neurons causes neurodegeneration and Lewy-like inclusions resembling human pale bodies. J Neurosci. 2008;28(33):8189-98.

Behl C, Davis JB, Lesley R, Schubert D. Hydrogen peroxide mediates amyloid beta protein toxicity. Cell. 1994;77(6):817-27.

Beister A, Kraus P, Kuhn W, Dose M, Weindl A, Gerlach M. The N-methyl-D-aspartate antagonist memantine retards progression of Huntington's disease. J Neural Transm Suppl. 2004(68):117-22.

Bence NF, Sampat RM, Kopito RR. Impairment of the ubiquitin-proteasome system by protein aggregation. Science. 2001;292(5521):1552-5.

Bennett EJ, Shaler TA, Woodman B, Ryu KY, Zaitseva TS, Becker CH, et al. Global changes to the ubiquitin system in Huntington's disease. Nature. 2007;448(7154):704-8.

Berger Z, Ravikumar B, Menzies FM, Oroz LG, Underwood BR, Pangalos MN, et al. Rapamycin alleviates toxicity of different aggregate-prone proteins. Hum Mol Genet. 2006;15(3):433-42.

Berke SJ, Chai Y, Marrs GL, Wen H, Paulson HL. Defining the role of ubiquitin-interacting motifs in the polyglutamine disease protein, ataxin-3. J Biol Chem. 2005;280(36):32026-34.

Berke SJ, Schmied FA, Brunt ER, Ellerby LM, Paulson HL. Caspase-mediated proteolysis of the polyglutamine disease protein ataxin-3. J Neurochem. 2004;89(4):908-18.

Bernier PJ, Bedard A, Vinet J, Levesque M, Parent A. Newly generated neurons in the amygdala and adjoining cortex of adult primates. Proc Natl Acad Sci U S A. 2002;99(17):11464-9.

Bettencourt C, Lima M. Machado-Joseph Disease: from first descriptions to new perspectives. Orphanet J Rare Dis. 2011;6:35.

Bettencourt C, Santos C, Kay T, Vasconcelos J, Lima M. Analysis of segregation patterns in Machado-Joseph disease pedigrees. J Hum Genet. 2008;53(10):920-3.

Bettencourt C, Santos C, Montiel R, Costa Mdo C, Cruz-Morales P, Santos LR, et al. Increased transcript diversity: novel splicing variants of Machado-Joseph disease gene (ATXN3). Neurogenetics. 2010;11(2):193-202.

Beyer PL, Palarino MY, Michalek D, Busenbark K, Koller WC. Weight change and body composition in patients with Parkinson's disease. J Am Diet Assoc. 1995;95(9):979-83.

Bi S, Robinson BM, Moran TH. Acute food deprivation and chronic food restriction differentially affect hypothalamic NPY mRNA expression. Am J Physiol Regul Integr Comp Physiol. 2003;285(5):R1030-6.

Bi X, Zhou J, Lynch G. Lysosomal protease inhibitors induce meganeurites and tangle-like structures in entorhinohippocampal regions vulnerable to Alzheimer's disease. Exp Neurol. 1999;158(2):312-27.

Bichelmeier U, Schmidt T, Hubener J, Boy J, Ruttiger L, Habig K, et al. Nuclear localization of ataxin-3 is required for the manifestation of symptoms in SCA3: in vivo evidence. J Neurosci. 2007;27(28):7418-28.

Bjorkqvist M, Wild EJ, Thiele J, Silvestroni A, Andre R, Lahiri N, et al. A novel pathogenic pathway of immune activation detectable before clinical onset in Huntington's disease. J Exp Med. 2008;205(8):1869-77.

Blagosklonny MV. Linking calorie restriction to longevity through sirtuins and autophagy: any role for TOR. Cell Death Dis. 2010;1:e12.

Block ML, Zecca L, Hong JS. Microglia-mediated neurotoxicity: uncovering the molecular mechanisms. Nat Rev Neurosci. 2007;8(1):57-69.

Blum-Degen D, Muller T, Kuhn W, Gerlach M, Przuntek H, Riederer P. Interleukin-1 beta and interleukin-6 are elevated in the cerebrospinal fluid of Alzheimer's and de novo Parkinson's disease patients. Neurosci Lett. 1995;202(1-2):17-20.

Boegman RJ, Smith Y, Parent A. Quinolinic acid does not spare striatal neuropeptide Y-immunoreactive neurons. Brain Res. 1987;415(1):178-82.

Boland B, Kumar A, Lee S, Platt FM, Wegiel J, Yu WH, et al. Autophagy induction and autophagosome clearance in neurons: relationship to autophagic pathology in Alzheimer's disease. J Neurosci. 2008;28(27):6926-37.

Borowsky B, Walker MW, Bard J, Weinshank RL, Laz TM, Vaysse P, et al. Molecular biology and pharmacology of multiple NPY Y5 receptor species homologs. Regul Pept. 1998;75-76:45-53.

Bovolenta R, Zucchini S, Paradiso B, Rodi D, Merigo F, Navarro Mora G, et al. Hippocampal FGF-2 and BDNF overexpression attenuates epileptogenesis-associated neuroinflammation and reduces spontaneous recurrent seizures. J Neuroinflammation. 2010;7:81.

Boy J, Schmidt T, Schumann U, Grasshoff U, Unser S, Holzmann C, et al. A transgenic mouse model of spinocerebellar ataxia type 3 resembling late disease onset and gender-specific instability of CAG repeats. Neurobiol Dis. 2010;37(2):284-93.

Boy J, Schmidt T, Wolburg H, Mack A, Nuber S, Bottcher M, et al. Reversibility of symptoms in a conditional mouse model of spinocerebellar ataxia type 3. Hum Mol Genet. 2009;18(22):4282-95.

Brady LS, Smith MA, Gold PW, Herkenham M. Altered expression of hypothalamic neuropeptide mRNAs in food-restricted and food-deprived rats. Neuroendocrinology. 1990;52(5):441-7.

Braga-Neto P, Felicio AC, Pedroso JL, Dutra LA, Bertolucci PH, Gabbai AA, et al. Clinical correlates of olfactory dysfunction in spinocerebellar ataxia type 3. Parkinsonism Relat Disord. 2011;17(5):353-6.

Brorson JR, Bindokas VP, Iwama T, Marcuccilli CJ, Chisholm JC, Miller RJ. The Ca2+ influx induced by beta-amyloid peptide 25-35 in cultured hippocampal neurons results from network excitation. J Neurobiol. 1995;26(3):325-38.

Browne SE, Bowling AC, MacGarvey U, Baik MJ, Berger SC, Muqit MM, et al. Oxidative damage and metabolic dysfunction in Huntington's disease: selective vulnerability of the basal ganglia. Ann Neurol. 1997;41(5):646-53.

Buchman AS, Wilson RS, Bienias JL, Shah RC, Evans DA, Bennett DA. Change in body mass index and risk of incident Alzheimer disease. Neurology. 2005;65(6):892-7.

Buhmann C, Bussopulos A, Oechsner M. Dopaminergic response in Parkinsonian phenotype of Machado-Joseph disease. Mov Disord. 2003;18(2):219-21.

Burkhoff A, Linemeyer DL, Salon JA. Distribution of a novel hypothalamic neuropeptide Y receptor gene and it's absence in rat. Brain Res Mol Brain Res. 1998;53(1-2):311-6.

Burnett B, Li F, Pittman RN. The polyglutamine neurodegenerative protein ataxin-3 binds polyubiquitylated proteins and has ubiquitin protease activity. Hum Mol Genet. 2003;12(23):3195-205.

Burt J, Alberto CO, Parsons MP, Hirasawa M. Local network regulation of orexin neurons in the lateral hypothalamus. Am J Physiol Regul Integr Comp Physiol. 2011;301(3):R572-80.

Caberlotto L, Fuxe K, Hurd YL. Characterization of NPY mRNA-expressing cells in the human brain: co-localization with Y2 but not Y1 mRNA in the cerebral cortex, hippocampus, amygdala, and striatum. J Chem Neuroanat. 2000;20(3-4):327-37.

Caberlotto L, Fuxe K, Rimland JM, Sedvall G, Hurd YL. Regional distribution of neuropeptide Y Y2 receptor messenger RNA in the human post mortem brain. Neuroscience. 1998;86(1):167-78.

Caberlotto L, Fuxe K, Sedvall G, Hurd YL. Localization of neuropeptide Y Y1 mRNA in the human brain: abundant expression in cerebral cortex and striatum. Eur J Neurosci. 1997;9(6):1212-25.

Cagnin A, Brooks DJ, Kennedy AM, Gunn RN, Myers R, Turkheimer FE, et al. In-vivo measurement of activated microglia in dementia. Lancet. 2001;358(9280):461-7.

Calabresi P, Mercuri NB, Sancesario G, Bernardi G. Electrophysiology of dopamine-denervated striatal neurons. Implications for Parkinson's disease. Brain. 1993;116 ( Pt 2):433-52.

Callen DJ, Black SE, Gao F, Caldwell CB, Szalai JP. Beyond the hippocampus: MRI volumetry confirms widespread limbic atrophy in AD. Neurology. 2001;57(9):1669-74.

Cancel G, Abbas N, Stevanin G, Durr A, Chneiweiss H, Neri C, et al. Marked phenotypic heterogeneity associated with expansion of a CAG repeat sequence at the spinocerebellar ataxia 3/Machado-Joseph disease locus. Am J Hum Genet. 1995;57(4):809-16.

Cannizzaro C, Tel BC, Rose S, Zeng BY, Jenner P. Increased neuropeptide Y mRNA expression in striatum in Parkinson's disease. Brain Res Mol Brain Res. 2003;110(2):169-76.

Caraceni T, Calderini G, Consolazione A, Riva E, Algeri S, Girotti F, et al. Biochemical aspects of Huntington's chorea. J Neurol Neurosurg Psychiatry. 1977;40(6):581-7.

Carvajal C, Dumont Y, Herzog H, Quirion R. Emotional behavior in aged neuropeptide Y (NPY) Y2 knockout mice. J Mol Neurosci. 2006;28(3):239-45.

Carvalho DR, La Rocque-Ferreira A, Rizzo IM, Imamura EU, Speck-Martins CE. Homozygosity enhances severity in spinocerebellar ataxia type 3. Pediatr Neurol. 2008;38(4):296-9.

Cataldo AM, Hamilton DJ, Nixon RA. Lysosomal abnormalities in degenerating neurons link neuronal compromise to senile plaque development in Alzheimer disease. Brain Res. 1994;640(1-2):68-80.

Cecchin CR, Pires AP, Rieder CR, Monte TL, Silveira I, Carvalho T, et al. Depressive symptoms in Machado-Joseph disease (SCA3) patients and their relatives. Community Genet. 2007;10(1):19-26.

Cemal CK, Carroll CJ, Lawrence L, Lowrie MB, Ruddle P, Al-Mahdawi S, et al. YAC transgenic mice carrying pathological alleles of the MJD1 locus exhibit a mild and slowly progressive cerebellar deficit. Hum Mol Genet. 2002;11(9):1075-94.

Cerda-Reverter JM, Larhammar D. Neuropeptide Y family of peptides: structure, anatomical expression, function, and molecular evolution. Biochem Cell Biol. 2000;78(3):371-92.

Chai Y, Berke SS, Cohen RE, Paulson HL. Poly-ubiquitin binding by the polyglutamine disease protein ataxin-3 links its normal function to protein surveillance pathways. J Biol Chem. 2004;279(5):3605-11.

Chai Y, Koppenhafer SL, Bonini NM, Paulson HL. Analysis of the role of heat shock protein (Hsp) molecular chaperones in polyglutamine disease. J Neurosci. 1999a;19(23):10338-47.

Chai Y, Koppenhafer SL, Shoesmith SJ, Perez MK, Paulson HL. Evidence for proteasome involvement in polyglutamine disease: localization to nuclear inclusions in SCA3/MJD and suppression of polyglutamine aggregation in vitro. Hum Mol Genet. 1999b;8(4):673-82.

Chai Y, Wu L, Griffin JD, Paulson HL. The role of protein composition in specifying nuclear inclusion formation in polyglutamine disease. J Biol Chem. 2001;276(48):44889-97.

Chan-Palay V, Asan E. Alterations in catecholamine neurons of the locus coeruleus in senile dementia of the Alzheimer type and in Parkinson's disease with and without dementia and depression. J Comp Neurol. 1989;287(3):373-92.

Chan-Palay V, Lang W, Allen YS, Haesler U, Polak JM. Cortical neurons immunoreactive with antisera against neuropeptide Y are altered in Alzheimer's-type dementia. J Comp Neurol. 1985;238(4):390-400.

Chan-Palay V, Lang W, Haesler U, Kohler C, Yasargil G. Distribution of altered hippocampal neurons and axons immunoreactive with antisera against neuropeptide Y in Alzheimer's-type dementia. J Comp Neurol. 1986;248(3):376-94.

Chari S, Quraishi SH, Jainer AK. Fluoxetine-induced exacerbation of chorea in Huntington's disease? A case report. Pharmacopsychiatry. 2003;36(1):41-3.

Charlett A, Dobbs RJ, Purkiss AG, Wright DJ, Peterson DW, Weller C, et al. Cortisol is higher in parkinsonism and associated with gait deficit. Acta Neurol Scand. 1998;97(2):77-85.

Checkoway H, Lundin JI, Kelada SN. Neurodegenerative diseases. IARC Sci Publ. 2011(163):407-19.

Chemelli RM, Willie JT, Sinton CM, Elmquist JK, Scammell T, Lee C, et al. Narcolepsy in orexin knockout mice: molecular genetics of sleep regulation. Cell. 1999;98(4):437-51.

Chen H, Zhang SM, Hernan MA, Willett WC, Ascherio A. Weight loss in Parkinson's disease. Ann Neurol. 2003;53(5):676-9.

Chen M, Ona VO, Li M, Ferrante RJ, Fink KB, Zhu S, et al. Minocycline inhibits caspase-1 and caspase-3 expression and delays mortality in a transgenic mouse model of Huntington disease. Nat Med. 2000;6(7):797-801.

Chen X, Tang TS, Tu H, Nelson O, Pook M, Hammer R, et al. Deranged calcium signaling and neurodegeneration in spinocerebellar ataxia type 3. J Neurosci. 2008;28(48):12713-24.

Cheng AV, Ferrier IN, Morris CM, Jabeen S, Sahgal A, McKeith IG, et al. Cortical serotonin-S2 receptor binding in Lewy body dementia, Alzheimer's and Parkinson's diseases. J Neurol Sci. 1991;106(1):50-5.

Cheng MF. Hypothalamic neurogenesis in the adult brain. Front Neuroendocrinol. 2013;34(3):167-78.

Chintawar S, Hourez R, Ravella A, Gall D, Orduz D, Rai M, et al. Grafting neural precursor cells promotes functional recovery in an SCA1 mouse model. J Neurosci. 2009;29(42):13126-35.

Chou AH, Yeh TH, Kuo YL, Kao YC, Jou MJ, Hsu CY, et al. Polyglutamine-expanded ataxin-3 activates mitochondrial apoptotic pathway by upregulating Bax and downregulating Bcl-xL. Neurobiol Dis. 2006;21(2):333-45.

Chou AH, Yeh TH, Ouyang P, Chen YL, Chen SY, Wang HL. Polyglutamine-expanded ataxin-3 causes cerebellar dysfunction of SCA3 transgenic mice by inducing transcriptional dysregulation. Neurobiol Dis. 2008;31(1):89-101.

Chouinard J. Dysphagia in Alzheimer disease: a review. J Nutr Health Aging. 2000;4(4):214-7.

Chow MK, Mackay JP, Whisstock JC, Scanlon MJ, Bottomley SP. Structural and functional analysis of the Josephin domain of the polyglutamine protein ataxin-3. Biochem Biophys Res Commun. 2004;322(2):387-94.

Christine CW, Starr PA, Larson PS, Eberling JL, Jagust WJ, Hawkins RA, et al. Safety and tolerability of putaminal AADC gene therapy for Parkinson disease. Neurology. 2009;73(20):1662-9.

Cicchetti F, Brownell AL, Williams K, Chen YI, Livni E, Isacson O. Neuroinflammation of the nigrostriatal pathway during progressive 6-OHDA dopamine degeneration in rats monitored by immunohistochemistry and PET imaging. Eur J Neurosci. 2002;15(6):991-8.

Ciechanover A, Brundin P. The ubiquitin proteasome system in neurodegenerative diseases: sometimes the chicken, sometimes the egg. Neuron. 2003;40(2):427-46.

Ciesielska A, Joniec I, Przybylkowski A, Gromadzka G, Kurkowska-Jastrzebska I, Czlonkowska A, et al. Dynamics of expression of the mRNA for cytokines and inducible nitric synthase in a murine model of the Parkinson's disease. Acta Neurobiol Exp (Wars). 2003;63(2):117-26.

Clark JT, Kalra PS, Crowley WR, Kalra SP. Neuropeptide Y and human pancreatic polypeptide stimulate feeding behavior in rats. Endocrinology. 1984;115(1):427-9.

Colmers WF, Klapstein GJ, Fournier A, St-Pierre S, Treherne KA. Presynaptic inhibition by neuropeptide Y in rat hippocampal slice in vitro is mediated by a Y2 receptor. Br J Pharmacol. 1991;102(1):41-4.

Corvino V, Marchese E, Giannetti S, Lattanzi W, Bonvissuto D, Biamonte F, et al. The neuroprotective and neurogenic effects of neuropeptide Y administration in an animal model of hippocampal neurodegeneration and temporal lobe epilepsy induced by trimethyltin. J Neurochem. 2012;122(2):415-26.

Corvino V, Marchese E, Podda MV, Lattanzi W, Giannetti S, Di Maria V, et al. The neurogenic effects of exogenous neuropeptide Y: early molecular events and long-lasting effects in the hippocampus of trimethyltin-treated rats. PLoS One. 2014;9(2):e88294.

Costa MdC, Paulson HL. Toward understanding Machado-Joseph disease. Prog Neurobiol. 2012;97(2):239-57.

Costantini LC, Bakowska JC, Breakefield XO, Isacson O. Gene therapy in the CNS. Gene Ther. 2000;7(2):93-109.

Coutinho P, Andrade C. Autosomal dominant system degeneration in Portuguese families of the Azores Islands. A new genetic disorder involving cerebellar, pyramidal, extrapyramidal and spinal cord motor functions. Neurology. 1978;28(7):703-9.

Coutinho P, Guimaraes A, Scaravilli F. The pathology of Machado-Joseph disease. Report of a possible homozygous case. Acta Neuropathol. 1982;58(1):48-54.

Crespi F. Influence of Neuropeptide Y and antidepressants upon cerebral monoamines involved in depression: an in vivo electrochemical study. Brain Res. 2011;1407:27-37.

Croce N, Ciotti MT, Gelfo F, Cortelli S, Federici G, Caltagirone C, et al. Neuropeptide Y protects rat cortical neurons against beta-amyloid toxicity and re-establishes synthesis and release of nerve growth factor. ACS Chem Neurosci. 2012;3(4):312-8.

Croce N, Gelfo F, Ciotti MT, Federici G, Caltagirone C, Bernardini S, et al. NPY modulates miR-30a-5p and BDNF in opposite direction in an in vitro model of Alzheimer disease: a possible role in neuroprotection? Mol Cell Biochem. 2013;376(1-2):189-95.

Cummings CJ, Mancini MA, Antalffy B, DeFranco DB, Orr HT, Zoghbi HY. Chaperone suppression of aggregation and altered subcellular proteasome localization imply protein misfolding in SCA1. Nat Genet. 1998;19(2):148-54.

Cummings CJ, Zoghbi HY. Fourteen and counting: unraveling trinucleotide repeat diseases. Hum Mol Genet. 2000;9(6):909-16.

Cvetanovic M, Ingram M, Orr H, Opal P. Early activation of microglia and astrocytes in mouse models of spinocerebellar ataxia type 1. Neuroscience. 2015;289:289-99.

Czlonkowska A, Kurkowska-Jastrzebska I. Inflammation and gliosis in neurological diseases--clinical implications. J Neuroimmunol. 2011;231(1-2):78-85.

D'Abreu A, Franca MC, Jr., Paulson HL, Lopes-Cendes I. Caring for Machado-Joseph disease: current understanding and how to help patients. Parkinsonism Relat Disord. 2010;16(1):2-7.

D'Angelo E, Casali S. Seeking a unified framework for cerebellar function and dysfunction: from circuit operations to cognition. Front Neural Circuits. 2012;6:116.

Dalrymple A, Wild EJ, Joubert R, Sathasivam K, Bjorkqvist M, Petersen A, et al. Proteomic profiling of plasma in Huntington's disease reveals neuroinflammatory activation and biomarker candidates. J Proteome Res. 2007;6(7):2833-40.

Davies CA, Morroll DR, Prinja D, Mann DM, Gibbs A. A quantitative assessment of somatostatin-like and neuropeptide Y-like immunostained cells in the frontal and temporal cortex of patients with Alzheimer's disease. J Neurol Sci. 1990;96(1):59-73.

Davis KL, Davis BM, Greenwald BS, Mohs RC, Mathe AA, Johns CA, et al. Cortisol and Alzheimer's disease, I: Basal studies. Am J Psychiatry. 1986;143(3):300-5.

Dawbarn D, De Quidt ME, Emson PC. Survival of basal ganglia neuropeptide Y-somatostatin neurones in Huntington's disease. Brain Res. 1985;340(2):251-60.

Dawson TM, Bredt DS, Fotuhi M, Hwang PM, Snyder SH. Nitric oxide synthase and neuronal NADPH diaphorase are identical in brain and peripheral tissues. Proc Natl Acad Sci U S A. 1991;88(17):7797-801.

Dayer AG, Cleaver KM, Abouantoun T, Cameron HA. New GABAergic interneurons in the adult neocortex and striatum are generated from different precursors. J Cell Biol. 2005;168(3):415-27.

de Almeida LP, Ross CA, Zala D, Aebischer P, Deglon N. Lentiviral-mediated delivery of mutant huntingtin in the striatum of rats induces a selective neuropathology modulated by polyglutamine repeat size, huntingtin expression levels, and protein length. J Neurosci. 2002;22(9):3473-83.

de Almeida LP, Zala D, Aebischer P, Deglon N. Neuroprotective effect of a CNTF-expressing lentiviral vector in the quinolinic acid rat model of Huntington's disease. Neurobiol Dis. 2001;8(3):433-46.

de Lau LM, Breteler MM. Epidemiology of Parkinson's disease. Lancet Neurol. 2006;5(6):525-35.

de Quidt ME, Emson PC. Distribution of neuropeptide Y-like immunoreactivity in the rat central nervous system--I. Radioimmunoassay and chromatographic characterisation. Neuroscience. 1986;18(3):527-43.

de Rezende TJ, D'Abreu A, Guimaraes RP, Lopes TM, Lopes-Cendes I, Cendes F, et al. Cerebral cortex involvement in Machado-Joseph disease. Eur J Neurol. 2015;22(2):277-83, e23-4.

Decressac M, Pain S, Chabeauti PY, Frangeul L, Thiriet N, Herzog H, et al. Neuroprotection by neuropeptide Y in cell and animal models of Parkinson's disease. Neurobiol Aging. 2012;33(9):2125-37.

Decressac M, Prestoz L, Veran J, Cantereau A, Jaber M, Gaillard A. Neuropeptide Y stimulates proliferation, migration and differentiation of neural precursors from the subventricular zone in adult mice. Neurobiol Dis. 2009;34(3):441-9.

Decressac M, Wright B, David B, Tyers P, Jaber M, Barker RA, et al. Exogenous neuropeptide Y promotes in vivo hippocampal neurogenesis. Hippocampus. 2011;21(3):233-8.

Decressac M, Wright B, Tyers P, Gaillard A, Barker RA. Neuropeptide Y modifies the disease course in the R6/2 transgenic model of Huntington's disease. Exp Neurol. 2010;226(1):24-32.

Deglon N, Hantraye P. Viral vectors as tools to model and treat neurodegenerative disorders. J Gene Med. 2005;7(5):530-9.

Del Bo R, Angeretti N, Lucca E, De Simoni MG, Forloni G. Reciprocal control of inflammatory cytokines, IL-1 and IL-6, and beta-amyloid production in cultures. Neurosci Lett. 1995;188(1):70-4.

Del Dotto P, Pavese N, Gambaccini G, Bernardini S, Metman LV, Chase TN, et al. Intravenous amantadine improves levadopa-induced dyskinesias: an acute double-blind placebo-controlled study. Mov Disord. 2001;16(3):515-20.

Dell'Anno MT, Caiazzo M, Leo D, Dvoretskova E, Medrihan L, Colasante G, et al. Remote control of induced dopaminergic neurons in parkinsonian rats. J Clin Invest. 2014;124(7):3215-29.

Diez M, Danner S, Frey P, Sommer B, Staufenbiel M, Wiederhold KH, et al. Neuropeptide alterations in the hippocampal formation and cortex of transgenic mice overexpressing beta-amyloid precursor protein (APP) with the Swedish double mutation (APP23). Neurobiol Dis. 2003;14(3):579-94.

Diez M, Koistinaho J, Kahn K, Games D, Hokfelt T. Neuropeptides in hippocampus and cortex in transgenic mice overexpressing V717F beta-amyloid precursor protein--initial observations. Neuroscience. 2000;100(2):259-86.

DiFiglia M, Sapp E, Chase KO, Davies SW, Bates GP, Vonsattel JP, et al. Aggregation of huntingtin in neuronal intranuclear inclusions and dystrophic neurites in brain. Science. 1997;277(5334):1990-3.

DiFiglia M, Sena-Esteves M, Chase K, Sapp E, Pfister E, Sass M, et al. Therapeutic silencing of mutant huntingtin with siRNA attenuates striatal and cortical neuropathology and behavioral deficits. Proc Natl Acad Sci U S A. 2007;104(43):17204-9.

Dinger MC, Bader JE, Kobor AD, Kretzschmar AK, Beck-Sickinger AG. Homodimerization of neuropeptide y receptors investigated by fluorescence resonance energy transfer in living cells. J Biol Chem. 2003;278(12):10562-71.

Djousse L, Knowlton B, Cupples LA, Marder K, Shoulson I, Myers RH. Weight loss in early stage of Huntington's disease. Neurology. 2002;59(9):1325-30.

Donaldson KM, Li W, Ching KA, Batalov S, Tsai CC, Joazeiro CA. Ubiquitin-mediated sequestration of normal cellular proteins into polyglutamine aggregates. Proc Natl Acad Sci U S A. 2003;100(15):8892-7.

Dong XX, Wang Y, Qin ZH. Molecular mechanisms of excitotoxicity and their relevance to pathogenesis of neurodegenerative diseases. Acta Pharmacol Sin. 2009;30(4):379-87.

dos Santos DF, Pedroso JL, Braga-Neto P, Silva GM, de Carvalho LB, Prado LB, et al. Excessive fragmentary myoclonus in Machado-Joseph disease. Sleep Med. 2014;15(3):355-8.

Doss-Pepe EW, Stenroos ES, Johnson WG, Madura K. Ataxin-3 Interactions with Rad23 and Valosin-Containing Protein and Its Associations with Ubiquitin Chains and the Proteasome Are Consistent with a Role in Ubiquitin-Mediated Proteolysis. Molecular and Cellular Biology. 2003;23(18):6469-83.

Douaud G, Gaura V, Ribeiro MJ, Lethimonnier F, Maroy R, Verny C, et al. Distribution of grey matter atrophy in Huntington's disease patients: a combined ROI-based and voxel-based morphometric study. Neuroimage. 2006;32(4):1562-75.

Duan W, Ross CA. Potential therapeutic targets for neurodegenerative diseases: lessons learned from calorie restriction. Curr Drug Targets. 2010;11(10):1281-92.

Duenas AM, Goold R, Giunti P. Molecular pathogenesis of spinocerebellar ataxias. Brain. 2006;129(Pt 6):1357-70.

Duff K, Paulsen JS, Beglinger LJ, Langbehn DR, Stout JC, Predict HDIotHSG. Psychiatric symptoms in Huntington's disease before diagnosis: the predict-HD study. Biol Psychiatry. 2007;62(12):1341-6.

Dumont Y, Jacques D, Bouchard P, Quirion R. Species differences in the expression and distribution of the neuropeptide Y Y1, Y2, Y4, and Y5 receptors in rodents, guinea pig, and primates brains. J Comp Neurol. 1998;402(3):372-84.

Duncan C, Papanikolaou T, Ellerby LM. Autophagy: polyQ toxic fragment turnover. Autophagy. 2010;6(2):312-4.

Durr A, Stevanin G, Cancel G, Duyckaerts C, Abbas N, Didierjean O, et al. Spinocerebellar ataxia 3 and Machado-Joseph disease: clinical, molecular, and neuropathological features. Ann Neurol. 1996;39(4):490-9.

Edvinsson L, Minthon L, Ekman R, Gustafson L. Neuropeptides in cerebrospinal fluid of patients with Alzheimer's disease and dementia with frontotemporal lobe degeneration. Dementia. 1993;4(3-4):167-71.

Edwards CM, Abusnana S, Sunter D, Murphy KG, Ghatei MA, Bloom SR. The effect of the orexins on food intake: comparison with neuropeptide Y, melanin-concentrating hormone and galanin. J Endocrinol. 1999;160(3):R7-12.

Ekdahl CT, Claasen JH, Bonde S, Kokaia Z, Lindvall O. Inflammation is detrimental for neurogenesis in adult brain. Proc Natl Acad Sci U S A. 2003;100(23):13632-7.

Emre M. Dementia associated with Parkinson's disease. Lancet Neurol. 2003;2(4):229-37.

Evers MM, Toonen LJ, van Roon-Mom WM. Ataxin-3 protein and RNA toxicity in spinocerebellar ataxia type 3: current insights and emerging therapeutic strategies. Mol Neurobiol. 2014;49(3):1513-31.

Evert BO, Araujo J, Vieira-Saecker AM, de Vos RA, Harendza S, Klockgether T, et al. Ataxin-3 represses transcription via chromatin binding, interaction with histone deacetylase 3, and histone deacetylation. J Neurosci. 2006a;26(44):11474-86.

Evert BO, Schelhaas J, Fleischer H, de Vos RA, Brunt ER, Stenzel W, et al. Neuronal intranuclear inclusions, dysregulation of cytokine expression and cell death in spinocerebellar ataxia type 3. Clin Neuropathol. 2006b;25(6):272-81.

Evert BO, Vogt IR, Kindermann C, Ozimek L, de Vos RA, Brunt ER, et al. Inflammatory genes are upregulated in expanded ataxin-3-expressing cell lines and spinocerebellar ataxia type 3 brains. J Neurosci. 2001;21(15):5389-96.

Fancellu R, Paridi D, Tomasello C, Panzeri M, Castaldo A, Genitrini S, et al. Longitudinal study of cognitive and psychiatric functions in spinocerebellar ataxia types 1 and 2. J Neurol. 2013;260(12):3134-43.

Farrer LA, Meaney FJ. An anthropometric assessment of Huntington's disease patients and families. Am J Phys Anthropol. 1985;67(3):185-94.

Fergusson J, Landon M, Lowe J, Dawson SP, Layfield R, Hanger DP, et al. Pathological lesions of Alzheimer's disease and dementia with Lewy bodies brains exhibit immunoreactivity to an ATPase that is a regulatory subunit of the 26S proteasome. Neurosci Lett. 1996;219(3):167-70.

Fernandes HB, Baimbridge KG, Church J, Hayden MR, Raymond LA. Mitochondrial sensitivity and altered calcium handling underlie enhanced NMDA-induced apoptosis in YAC128 model of Huntington's disease. J Neurosci. 2007;27(50):13614-23.

Ferreira R, Santos T, Cortes L, Cochaud S, Agasse F, Silva AP, et al. Neuropeptide Y inhibits interleukin-1 beta-induced microglia motility. J Neurochem. 2012;120(1):93-105.

Ferreira R, Santos T, Viegas M, Cortes L, Bernardino L, Vieira OV, et al. Neuropeptide Y inhibits interleukin-1beta-induced phagocytosis by microglial cells. J Neuroinflammation. 2011;8:169.

Ferreira R, Xapelli S, Santos T, Silva AP, Cristovao A, Cortes L, et al. Neuropeptide Y modulation of interleukin-1beta (IL-1b)-induced nitric oxide production in microglia. J Biol Chem. 2010;285(53):41921-34.

Ferrer I, Goutan E, Marin C, Rey MJ, Ribalta T. Brain-derived neurotrophic factor in Huntington disease. Brain Res. 2000;866(1-2):257-61.

Figueredo-Cardenas G, Morello M, Sancesario G, Bernardi G, Reiner A. Colocalization of somatostatin, neuropeptide Y, neuronal nitric oxide synthase and NADPH-diaphorase in striatal interneurons in rats. Brain Res. 1996;735(2):317-24.

Folstein MF, Whitehouse PJ. Cognitive impairment of Alzheimer disease. Neurobehav Toxicol Teratol. 1983;5(6):631-4.

Franich NR, Fitzsimons HL, Fong DM, Klugmann M, During MJ, Young D. AAV vector-mediated RNAi of mutant huntingtin expression is neuroprotective in a novel genetic rat model of Huntington's disease. Mol Ther. 2008;16(5):947-56.

Freeman W, Wszolek Z. Botulinum toxin type A for treatment of spasticity in spinocerebellar ataxia type 3 (Machado-Joseph disease). Mov Disord. 2005;20(5):644.

Frommberger UH, Bauer J, Haselbauer P, Fraulin A, Riemann D, Berger M. Interleukin-6-(IL-6) plasma levels in depression and schizophrenia: comparison between the acute state and after remission. Eur Arch Psychiatry Clin Neurosci. 1997;247(4):228-33.

Fronczek R, van Geest S, Frolich M, Overeem S, Roelandse FW, Lammers GJ, et al. Hypocretin (orexin) loss in Alzheimer's disease. Neurobiol Aging. 2012;33(8):1642-50.

Fujigasaki H, Uchihara T, Koyano S, Iwabuchi K, Yagishita S, Makifuchi T, et al. Ataxin-3 is translocated into the nucleus for the formation of intranuclear inclusions in normal and Machado-Joseph disease brains. Exp Neurol. 2000;165(2):248-56.

Furtinger S, Pirker S, Czech T, Baumgartner C, Ransmayr G, Sperk G. Plasticity of Y1 and Y2 receptors and neuropeptide Y fibers in patients with temporal lobe epilepsy. J Neurosci. 2001;21(15):5804-12.

Gabery S, Georgiou-Karistianis N, Lundh SH, Cheong RY, Churchyard A, Chua P, et al. Volumetric analysis of the hypothalamus in Huntington Disease using 3T MRI: the IMAGE-HD Study. PLoS One. 2015;10(2):e0117593.

Gabery S, Murphy K, Schultz K, Loy CT, McCusker E, Kirik D, et al. Changes in key hypothalamic neuropeptide populations in Huntington disease revealed by neuropathological analyses. Acta Neuropathol. 2010;120(6):777-88.

Garcia BG, Neely MD, Deutch AY. Cortical regulation of striatal medium spiny neuron dendritic remodeling in parkinsonism: modulation of glutamate release reverses dopamine depletion-induced dendritic spine loss. Cereb Cortex. 2010;20(10):2423-32.

Garden GA, Moller T. Microglia biology in health and disease. J Neuroimmune Pharmacol. 2006;1(2):127-37.

Gaspar C, Lopes-Cendes I, Hayes S, Goto J, Arvidsson K, Dias A, et al. Ancestral origins of the Machado-Joseph disease mutation: a worldwide haplotype study. Am J Hum Genet. 2001;68(2):523-8.

Gehlert DR. Introduction to the reviews on neuropeptide Y. Neuropeptides. 2004;38(4):135-40.

Gehlert DR, Schober DA, Morin M, Berglund MM. Co-expression of neuropeptide Y Y1 and Y5 receptors results in heterodimerization and altered functional properties. Biochem Pharmacol. 2007;74(11):1652-64.

Gendall KA, Kaye WH, Altemus M, McConaha CW, La Via MC. Leptin, neuropeptide Y, and peptide YY in long-term recovered eating disorder patients. Biol Psychiatry. 1999;46(2):292-9.

Gerald C, Walker MW, Criscione L, Gustafson EL, Batzl-Hartmann C, Smith KE, et al. A receptor subtype involved in neuropeptide-Y-induced food intake. Nature. 1996;382(6587):168-71.

Gerhard A, Pavese N, Hotton G, Turkheimer F, Es M, Hammers A, et al. In vivo imaging of microglial activation with [11C](R)-PK11195 PET in idiopathic Parkinson's disease. Neurobiol Dis. 2006;21(2):404-12.

Gillette-Guyonnet S, Nourhashemi F, Andrieu S, de Glisezinski I, Ousset PJ, Riviere D, et al. Weight loss in Alzheimer disease. Am J Clin Nutr. 2000;71(2):637S-42S.

Ginn SL, Alexander IE, Edelstein ML, Abedi MR, Wixon J. Gene therapy clinical trials worldwide to 2012 - an update. J Gene Med. 2013;15(2):65-77.

Global Burden of Disease Study C. Global, regional, and national incidence, prevalence, and years lived with disability for 301 acute and chronic diseases and injuries in 188 countries, 1990-2013: a systematic analysis for the Global Burden of Disease Study 2013. Lancet. 2015.

Goncalves J, Ribeiro CF, Malva JO, Silva AP. Protective role of neuropeptide Y Y(2) receptors in cell death and microglial response following methamphetamine injury. Eur J Neurosci. 2012;36(9):3173-83.

Goncalves N, Simoes AT, Cunha RA, de Almeida LP. Caffeine and adenosine A(2A) receptor inactivation decrease striatal neuropathology in a lentiviral-based model of Machado-Joseph disease. Ann Neurol. 2013;73(5):655-66.

Gordon RJ, Tattersfield AS, Vazey EM, Kells AP, McGregor AL, Hughes SM, et al. Temporal profile of subventricular zone progenitor cell migration following quinolinic acid-induced striatal cell loss. Neuroscience. 2007;146(4):1704-18.

Gotham AM, Brown RG, Marsden CD. Depression in Parkinson's disease: a quantitative and qualitative analysis. J Neurol Neurosurg Psychiatry. 1986;49(4):381-9.

Goti D, Katzen SM, Mez J, Kurtis N, Kiluk J, Ben-Haiem L, et al. A mutant ataxin-3 putative-cleavage fragment in brains of Machado-Joseph disease patients and transgenic mice is cytotoxic above a critical concentration. J Neurosci. 2004;24(45):10266-79.

Gould E, Vail N, Wagers M, Gross CG. Adult-generated hippocampal and neocortical neurons in macaques have a transient existence. Proc Natl Acad Sci U S A. 2001;98(19):10910-7.

Gray CW, Patel AJ. Neurodegeneration mediated by glutamate and beta-amyloid peptide: a comparison and possible interaction. Brain Res. 1995;691(1-2):169-79.

Greengard P, Allen PB, Nairn AC. Beyond the dopamine receptor: the DARPP-32/protein phosphatase-1 cascade. Neuron. 1999;23(3):435-47.

Groves JO. Is it time to reassess the BDNF hypothesis of depression? Mol Psychiatry. 2007;12(12):1079-88.

Guerin O, Andrieu S, Schneider SM, Milano M, Boulahssass R, Brocker P, et al. Different modes of weight loss in Alzheimer disease: a prospective study of 395 patients. Am J Clin Nutr. 2005;82(2):435-41.

Gurevich D, Siegel B, Dumlao M, Perl E, Chaitin P, Bagne C, et al. HPA axis responsivity to dexamethasone and cognitive impairment in dementia. Prog Neuropsychopharmacol Biol Psychiatry. 1990;14(3):297-308.

Gustafson EL, Smith KE, Durkin MM, Walker MW, Gerald C, Weinshank R, et al. Distribution of the neuropeptide Y Y2 receptor mRNA in rat central nervous system. Brain Res Mol Brain Res. 1997;46(1-2):223-35.

Hamilton JM, Salmon DP, Corey-Bloom J, Gamst A, Paulsen JS, Jerkins S, et al. Behavioural abnormalities contribute to functional decline in Huntington's disease. J Neurol Neurosurg Psychiatry. 2003;74(1):120-2.

Hansel DE, Eipper BA, Ronnett GV. Neuropeptide Y functions as a neuroproliferative factor. Nature. 2001;410(6831):940-4.

Hansen M, Chandra A, Mitic LL, Onken B, Driscoll M, Kenyon C. A Role for Autophagy Genes in the Extension of Lifespan by Dietary Restriction in C. elegans. PLoS Genetics. 2005;preprint(2008):e24.

Hara J, Beuckmann CT, Nambu T, Willie JT, Chemelli RM, Sinton CM, et al. Genetic ablation of orexin neurons in mice results in narcolepsy, hypophagia, and obesity. Neuron. 2001;30(2):345-54.

Hara T, Nakamura K, Matsui M, Yamamoto A, Nakahara Y, Suzuki-Migishima R, et al. Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. Nature. 2006;441(7095):885-9.

Hardy J, Cookson MR, Singleton A. Genes and parkinsonism. Lancet Neurol. 2003;2(4):221-8.

Harkany T, Abraham I, Timmerman W, Laskay G, Toth B, Sasvari M, et al. beta-amyloid neurotoxicity is mediated by a glutamate-triggered excitotoxic cascade in rat nucleus basalis. Eur J Neurosci. 2000;12(8):2735-45.

Harper SQ, Staber PD, He X, Eliason SL, Martins IH, Mao Q, et al. RNA interference improves motor and neuropathological abnormalities in a Huntington's disease mouse model. Proc Natl Acad Sci U S A. 2005;102(16):5820-5.

Harris GM, Dodelzon K, Gong L, Gonzalez-Alegre P, Paulson HL. Splice isoforms of the polyglutamine disease protein ataxin-3 exhibit similar enzymatic yet different aggregation properties. PLoS One. 2010;5(10):e13695.

Hawkes C. Olfaction in neurodegenerative disorder. Mov Disord. 2003;18(4):364-72.

Haynes AC, Jackson B, Chapman H, Tadayyon M, Johns A, Porter RA, et al. A selective orexin-1 receptor antagonist reduces food consumption in male and female rats. Regul Pept. 2000;96(1-2):45-51.

Heilig M, Sjogren M, Blennow K, Ekman R, Wallin A. Cerebrospinal fluid neuropeptides in Alzheimer's disease and vascular dementia. Biol Psychiatry. 1995;38(4):210-6.

Hendry SH, Jones EG, DeFelipe J, Schmechel D, Brandon C, Emson PC. Neuropeptide-containing neurons of the cerebral cortex are also GABAergic. Proc Natl Acad Sci U S A. 1984;81(20):6526-30.

Heng MY, Detloff PJ, Paulson HL, Albin RL. Early alterations of autophagy in Huntington disease-like mice. Autophagy. 2010;6(8):1206-8.

Heuser IJ, Chase TN, Mouradian MM. The limbic-hypothalamic-pituitary-adrenal axis in Huntington's disease. Biol Psychiatry. 1991;30(9):943-52.

Hock C, Heese K, Hulette C, Rosenberg C, Otten U. Region-specific neurotrophin imbalances in Alzheimer disease: decreased levels of brain-derived neurotrophic factor and increased levels of nerve growth factor in hippocampus and cortical areas. Arch Neurol. 2000;57(6):846-51.

Holmberg CI, Staniszewski KE, Mensah KN, Matouschek A, Morimoto RI. Inefficient degradation of truncated polyglutamine proteins by the proteasome. EMBO J. 2004;23(21):4307-18.

Holmberg M, Duyckaerts C, Durr A, Cancel G, Gourfinkel-An I, Damier P, et al. Spinocerebellar ataxia type 7 (SCA7): a neurodegenerative disorder with neuronal intranuclear inclusions. Hum Mol Genet. 1998;7(5):913-8.

Horimoto Y, Matsumoto M, Akatsu H, Kojima A, Yoshida M, Nokura K, et al. Longitudinal study on MRI intensity changes of Machado-Joseph disease: correlation between MRI findings and neuropathological changes. J Neurol. 2011;258(9):1657-64.

Hornykiewicz O. Parkinson's disease and its chemotherapy. Biochem Pharmacol. 1975;24(10):1061-5.

Hourez R, Servais L, Orduz D, Gall D, Millard I, de Kerchove d'Exaerde A, et al. Aminopyridines correct early dysfunction and delay neurodegeneration in a mouse model of spinocerebellar ataxia type 1. J Neurosci. 2011;31(33):11795-807.

Howell OW, Scharfman HE, Herzog H, Sundstrom LE, Beck-Sickinger A, Gray WP. Neuropeptide Y is neuroproliferative for post-natal hippocampal precursor cells. J Neurochem. 2003;86(3):646-59.

Hubener J, Riess O. Polyglutamine-induced neurodegeneration in SCA3 is not mitigated by non-expanded ataxin-3: conclusions from double-transgenic mouse models. Neurobiol Dis. 2010;38(1):116-24.

Hubener J, Vauti F, Funke C, Wolburg H, Ye Y, Schmidt T, et al. N-terminal ataxin-3 causes neurological symptoms with inclusions, endoplasmic reticulum stress and ribosomal dislocation. Brain. 2011;134(Pt 7):1925-42.

Hult Lundh S, Nilsson N, Soylu R, Kirik D, Petersen A. Hypothalamic expression of mutant huntingtin contributes to the development of depressive-like behavior in the BAC transgenic mouse model of Huntington's disease. Hum Mol Genet. 2013;22(17):3485-97.

Hult S, Soylu R, Bjorklund T, Belgardt BF, Mauer J, Bruning JC, et al. Mutant huntingtin causes metabolic imbalance by disruption of hypothalamic neurocircuits. Cell Metab. 2011;13(4):428-39.

Huntington Study G. Tetrabenazine as antichorea therapy in Huntington disease: a randomized controlled trial. Neurology. 2006;66(3):366-72.

Ikeda H, Yamaguchi M, Sugai S, Aze Y, Narumiya S, Kakizuka A. Expanded polyglutamine in the Machado-Joseph disease protein induces cell death in vitro and in vivo. Nat Genet. 1996;13(2):196-202.

Ilg W, Timmann D. Gait ataxia--specific cerebellar influences and their rehabilitation. Mov Disord. 2013;28(11):1566-75.

Ishikawa K, Fujigasaki H, Saegusa H, Ohwada K, Fujita T, Iwamoto H, et al. Abundant expression and cytoplasmic aggregations of [alpha]1A voltage-dependent calcium channel protein associated with neurodegeneration in spinocerebellar ataxia type 6. Hum Mol Genet. 1999;8(7):1185-93.

Isono C, Hirano M, Sakamoto H, Ueno S, Kusunoki S, Nakamura Y. Differences in dysphagia between spinocerebellar ataxia type 3 and type 6. Dysphagia. 2013;28(3):413-8.

Jaeger PA, Pickford F, Sun CH, Lucin KM, Masliah E, Wyss-Coray T. Regulation of amyloid precursor protein processing by the Beclin 1 complex. PLoS One. 2010;5(6):e11102.

Jana NR, Dikshit P, Goswami A, Kotliarova S, Murata S, Tanaka K, et al. Co-chaperone CHIP associates with expanded polyglutamine protein and promotes their degradation by proteasomes. J Biol Chem. 2005;280(12):11635-40.

Jana NR, Zemskov EA, Wang G, Nukina N. Altered proteasomal function due to the expression of polyglutamine-expanded truncated N-terminal huntingtin induces apoptosis by caspase activation through mitochondrial cytochrome c release. Hum Mol Genet. 2001;10(10):1049-59.

Jellinger KA. Pathology of Parkinson's disease. Changes other than the nigrostriatal pathway. Mol Chem Neuropathol. 1991;14(3):153-97.

Jia K, Hart AC, Levine B. Autophagy genes protect against disease caused by polyglutamine expansion proteins in Caenorhabditis elegans. Autophagy. 2007;3(1):21-5.

Johnson DK, Wilkins CH, Morris JC. Accelerated weight loss may precede diagnosis in Alzheimer disease. Arch Neurol. 2006;63(9):1312-7.

Johnson EM, Craig ET, Yeh HH. TrkB is necessary for pruning at the climbing fibre-Purkinje cell synapse in the developing murine cerebellum. J Physiol. 2007;582(Pt 2):629-46.

Jung J, Xu K, Lessing D, Bonini NM. Preventing Ataxin-3 protein cleavage mitigates degeneration in a Drosophila model of SCA3. Hum Mol Genet. 2009;18(24):4843-52.

Kagel MC, Leopold NA. Dysphagia in Huntington's disease: a 16-year retrospective. Dysphagia. 1992;7(2):106-14.

Kalra SP, Dube MG, Sahu A, Phelps CP, Kalra PS. Neuropeptide Y secretion increases in the paraventricular nucleus in association with increased appetite for food. Proc Natl Acad Sci U S A. 1991;88(23):10931-5.

Kanatani A, Mashiko S, Murai N, Sugimoto N, Ito J, Fukuroda T, et al. Role of the Y1 receptor in the regulation of neuropeptide Y-mediated feeding: comparison of wild-type, Y1 receptor-deficient, and Y5 receptor-deficient mice. Endocrinology. 2000;141(3):1011-6.

Kasanuki K, Iseki E, Kondo D, Fujishiro H, Minegishi M, Sato K, et al. Neuropathological investigation of hypocretin expression in brains of dementia with Lewy bodies. Neurosci Lett. 2014;569:68-73.

Kask A, Harro J, von Horsten S, Redrobe JP, Dumont Y, Quirion R. The neurocircuitry and receptor subtypes mediating anxiolytic-like effects of neuropeptide Y. Neurosci Biobehav Rev. 2002;26(3):259-83.

Kassubek J, Juengling FD, Kioschies T, Henkel K, Karitzky J, Kramer B, et al. Topography of cerebral atrophy in early Huntington's disease: a voxel based morphometric MRI study. J Neurol Neurosurg Psychiatry. 2004;75(2):213-20.

Kastin AJ, Akerstrom V. Nonsaturable entry of neuropeptide Y into brain. Am J Physiol. 1999;276(3 Pt 1):E479-82.

Kawaguchi Y, Okamoto T, Taniwaki M, Aizawa M, Inoue M, Katayama S, et al. CAG expansions in a novel gene for Machado-Joseph disease at chromosome 14q32.1. Nat Genet. 1994;8(3):221-8.

Kaye WH, Berrettini W, Gwirtsman H, George DT. Altered cerebrospinal fluid neuropeptide Y and peptide YY immunoreactivity in anorexia and bulimia nervosa. Arch Gen Psychiatry. 1990;47(6):548-56.

Kazachkova N, Raposo M, Montiel R, Cymbron T, Bettencourt C, Silva-Fernandes A, et al. Patterns of mitochondrial DNA damage in blood and brain tissues of a transgenic mouse model of Machado-Joseph disease. Neurodegener Dis. 2013;11(4):206-14.

Keck S, Nitsch R, Grune T, Ullrich O. Proteasome inhibition by paired helical filament-tau in brains of patients with Alzheimer's disease. J Neurochem. 2003;85(1):115-22.

Keller JN, Hanni KB, Markesbery WR. Impaired proteasome function in Alzheimer's disease. J Neurochem. 2000;75(1):436-9.

Kerkerian L, Bosler O, Pelletier G, Nieoullon A. Striatal neuropeptide Y neurones are under the influence of the nigrostriatal dopaminergic pathway: immunohistochemical evidence. Neurosci Lett. 1986;66(1):106-12.

Kerkerian L, Salin P, Nieoullon A. Pharmacological characterization of dopaminergic influence on expression of neuropeptide Y immunoreactivity by rat striatal neurons. Neuroscience. 1988;26(3):809-17.

Khan LA, Bauer PO, Miyazaki H, Lindenberg KS, Landwehrmeyer BG, Nukina N. Expanded polyglutamines impair synaptic transmission and ubiquitin-proteasome system in Caenorhabditis elegans. J Neurochem. 2006;98(2):576-87.

Kim S-J, Kim T-S, Kim IY, Hong S, Rhim H, Kang S. Polyglutamine-expanded ataxin-1 recruits Cu/Zn-superoxide dismutase into the nucleus of HeLa cells. Biochemical and Biophysical Research Communications. 2003;307(3):660-5.

Kim YT, Shin SM, Lee WY, Kim GM, Jin DK. Expression of expanded polyglutamine protein induces behavioral changes in Drosophila (polyglutamine-induced changes in Drosophila). Cell Mol Neurobiol. 2004;24(1):109-22.

Kingma EM, van Duijn E, Timman R, van der Mast RC, Roos RA. Behavioural problems in Huntington's disease using the Problem Behaviours Assessment. Gen Hosp Psychiatry. 2008;30(2):155-61.

Kirik D, Annett LE, Burger C, Muzyczka N, Mandel RJ, Bjorklund A. Nigrostriatal alpha-synucleinopathy induced by viral vector-mediated overexpression of human alpha-synuclein: a new primate model of Parkinson's disease. Proc Natl Acad Sci U S A. 2003;100(5):2884-9.

Kirik D, Rosenblad C, Burger C, Lundberg C, Johansen TE, Muzyczka N, et al. Parkinson-like neurodegeneration induced by targeted overexpression of alpha-synuclein in the nigrostriatal system. J Neurosci. 2002;22(7):2780-91.

Kish SJ, Tong J, Hornykiewicz O, Rajput A, Chang LJ, Guttman M, et al. Preferential loss of serotonin markers in caudate versus putamen in Parkinson's disease. Brain. 2008;131(Pt 1):120-31.

Klockgether T, Skalej M, Wedekind D, Luft AR, Welte D, Schulz JB, et al. Autosomal dominant cerebellar ataxia type I. MRI-based volumetry of posterior fossa structures and basal ganglia in spinocerebellar ataxia types 1, 2 and 3. Brain. 1998;121 ( Pt 9):1687-93.

Kloster E, Saft C, Akkad DA, Epplen JT, Arning L. Association of age at onset in Huntington disease with functional promoter variations in NPY and NPY2R. J Mol Med (Berl). 2014;92(2):177-84.

Knudsen S, Gammeltoft S, Jennum PJ. Rapid eye movement sleep behaviour disorder in patients with narcolepsy is associated with hypocretin-1 deficiency. Brain. 2010;133(Pt 2):568-79.

Koch P, Breuer P, Peitz M, Jungverdorben J, Kesavan J, Poppe D, et al. Excitation-induced ataxin-3 aggregation in neurons from patients with Machado-Joseph disease. Nature. 2011;480(7378):543-6.

Koide S, Onishi H, Hashimoto H, Kai T, Yamagami S. Plasma neuropeptide Y is reduced in patients with Alzheimer's disease. Neurosci Lett. 1995;198(2):149-51.

Kokoeva MV, Yin H, Flier JS. Evidence for constitutive neural cell proliferation in the adult murine hypothalamus. J Comp Neurol. 2007;505(2):209-20.

Komatsu M, Waguri S, Chiba T, Murata S, Iwata J, Tanida I, et al. Loss of autophagy in the central nervous system causes neurodegeneration in mice. Nature. 2006;441(7095):880-4.

Konno A, Shuvaev AN, Miyake N, Miyake K, Iizuka A, Matsuura S, et al. Mutant ataxin-3 with an abnormally expanded polyglutamine chain disrupts dendritic development and metabotropic glutamate receptor signaling in mouse cerebellar Purkinje cells. Cerebellum. 2014;13(1):29-41.

Kordower JH, Emborg ME, Bloch J, Ma SY, Chu Y, Leventhal L, et al. Neurodegeneration prevented by lentiviral vector delivery of GDNF in primate models of Parkinson's disease. Science. 2000;290(5492):767-73.

Kornstein SG, Schneider RK. Clinical features of treatment-resistant depression. J Clin Psychiatry. 2001;62 Suppl 16:18-25.

Kowall NW, Ferrante RJ, Beal MF, Richardson EP, Jr., Sofroniew MV, Cuello AC, et al. Neuropeptide Y, somatostatin, and reduced nicotinamide adenine dinucleotide phosphate diaphorase in the human striatum: a combined immunocytochemical and enzyme histochemical study. Neuroscience. 1987;20(3):817-28.

Koyano S, Uchihara T, Fujigasaki H, Nakamura A, Yagishita S, Iwabuchi K. Neuronal intranuclear inclusions in spinocerebellar ataxia type 2: triple-labeling immunofluorescent study. Neurosci Lett. 1999;273(2):117-20.

Kumar U. Characterization of striatal cultures with the effect of QUIN and NMDA. Neurosci Res. 2004;49(1):29-38.

Kuopio AM, Marttila RJ, Helenius H, Toivonen M, Rinne UK. The quality of life in Parkinson's disease. Mov Disord. 2000;15(2):216-23.

Kurkowska-Jastrzebska I, Babiuch M, Joniec I, Przybylkowski A, Czlonkowski A, Czlonkowska A. Indomethacin protects against neurodegeneration caused by MPTP intoxication in mice. Int Immunopharmacol. 2002;2(8):1213-8.

Kurkowska-Jastrzebska I, Litwin T, Joniec I, Ciesielska A, Przybylkowski A, Czlonkowski A, et al. Dexamethasone protects against dopaminergic neurons damage in a mouse model of Parkinson's disease. Int Immunopharmacol. 2004;4(10-11):1307-18.

La Spada AR, Wilson EM, Lubahn DB, Harding AE, Fischbeck KH. Androgen receptor gene mutations in X-linked spinal and bulbar muscular atrophy. Nature. 1991;352(6330):77-9.

Laco MN, Oliveira CR, Paulson HL, Rego AC. Compromised mitochondrial complex II in models of Machado-Joseph disease. Biochim Biophys Acta. 2012;1822(2):139-49.

Langston JW, Forno LS. The hypothalamus in Parkinson disease. Ann Neurol. 1978;3(2):129-33.

Larhammar D. Evolution of neuropeptide Y, peptide YY and pancreatic polypeptide. Regul Pept. 1996;62(1):1-11.

Larhammar D, Wraith A, Berglund MM, Holmberg SK, Lundell I. Origins of the many NPY-family receptors in mammals. Peptides. 2001;22(3):295-307.

Lau A, Tymianski M. Glutamate receptors, neurotoxicity and neurodegeneration. Pflugers Arch. 2010;460(2):525-42.

Laukova M, Alaluf LG, Serova LI, Arango V, Sabban EL. Early intervention with intranasal NPY prevents single prolonged stress-triggered impairments in hypothalamus and ventral hippocampus in male rats. Endocrinology. 2014;155(10):3920-33.

Lauwers E, Debyser Z, Van Dorpe J, De Strooper B, Nuttin B, Baekelandt V. Neuropathology and neurodegeneration in rodent brain induced by lentiviral vector-mediated overexpression of alpha-synuclein. Brain Pathol. 2003;13(3):364-72.

Leblhuber F, Peichl M, Neubauer C, Reisecker F, Steinparz FX, Windhager E, et al. Serum dehydroepiandrosterone and cortisol measurements in Huntington's chorea. J Neurol Sci. 1995;132(1):76-9.

Lee HJ, Khoshaghideh F, Patel S, Lee SJ. Clearance of alpha-synuclein oligomeric intermediates via the lysosomal degradation pathway. J Neurosci. 2004;24(8):1888-96.

Leentjens AF, Van den Akker M, Metsemakers JF, Lousberg R, Verhey FR. Higher incidence of depression preceding the onset of Parkinson's disease: a register study. Mov Disord. 2003;18(4):414-8.

Levine AS, Morley JE. Neuropeptide Y: a potent inducer of consummatory behavior in rats. Peptides. 1984;5(6):1025-9.

Levine MS, Klapstein GJ, Koppel A, Gruen E, Cepeda C, Vargas ME, et al. Enhanced sensitivity to N-methyl-D-aspartate receptor activation in transgenic and knockin mouse models of Huntington's disease. J Neurosci Res. 1999;58(4):515-32.

Lima L, Coutinho P. Clinical criteria for diagnosis of Machado-Joseph disease: report of a non-Azorena Portuguese family. Neurology. 1980;30(3):319-22.

Lima M, Mayer FM, Coutinho P, Abade A. Origins of a mutation: population genetics of Machado-Joseph disease in the Azores (Portugal). Hum Biol. 1998;70(6):1011-23.

Lin S, Boey D, Herzog H. NPY and Y receptors: lessons from transgenic and knockout models. Neuropeptides. 2004;38(4):189-200.

Lin X, Antalffy B, Kang D, Orr HT, Zoghbi HY. Polyglutamine expansion down-regulates specific neuronal genes before pathologic changes in SCA1. Nat Neurosci. 2000;3(2):157-63.

Lo Bianco C, Ridet JL, Schneider BL, Deglon N, Aebischer P. alpha -Synucleinopathy and selective dopaminergic neuron loss in a rat lentiviral-based model of Parkinson's disease. Proc Natl Acad Sci U S A. 2002;99(16):10813-8.

Loskutova N, Honea RA, Brooks WM, Burns JM. Reduced limbic and hypothalamic volumes correlate with bone density in early Alzheimer's disease. J Alzheimers Dis. 2010;20(1):313-22.

Lucetti C, Gambaccini G, Bernardini S, Dell'Agnello G, Petrozzi L, Rossi G, et al. Amantadine in Huntington's disease: open-label video-blinded study. Neurol Sci. 2002;23 Suppl 2:S83-4.

Lundell I, Blomqvist AG, Berglund MM, Schober DA, Johnson D, Statnick MA, et al. Cloning of a human receptor of the NPY receptor family with high affinity for pancreatic polypeptide and peptide YY. J Biol Chem. 1995;270(49):29123-8.

Luzzati F, De Marchis S, Fasolo A, Peretto P. Neurogenesis in the caudate nucleus of the adult rabbit. J Neurosci. 2006;26(2):609-21.

Maccioni RB, Rojo LE, Fernandez JA, Kuljis RO. The role of neuroimmunomodulation in Alzheimer's disease. Ann N Y Acad Sci. 2009;1153:240-6.

Macia F, Perlemoine C, Coman I, Guehl D, Burbaud P, Cuny E, et al. Parkinson's disease patients with bilateral subthalamic deep brain stimulation gain weight. Mov Disord. 2004;19(2):206-12.

Maciel P, Costa MC, Ferro A, Rousseau M, Santos CS, Gaspar C, et al. Improvement in the molecular diagnosis of Machado-Joseph disease. Arch Neurol. 2001;58(11):1821-7.

Maciel P, Gaspar C, DeStefano AL, Silveira I, Coutinho P, Radvany J, et al. Correlation between CAG repeat length and clinical features in Machado-Joseph disease. Am J Hum Genet. 1995;57(1):54-61.

Majumder S, Richardson A, Strong R, Oddo S. Inducing autophagy by rapamycin before, but not after, the formation of plaques and tangles ameliorates cognitive deficits. PLoS One. 2011;6(9):e25416.

Mandel S, Grunblatt E, Riederer P, Amariglio N, Jacob-Hirsch J, Rechavi G, et al. Gene expression profiling of sporadic Parkinson's disease substantia nigra pars compacta reveals impairment of ubiquitin-proteasome subunits, SKP1A, aldehyde dehydrogenase, and chaperone HSC-70. Ann N Y Acad Sci. 2005;1053:356-75.

Mandrusiak LM. Transglutaminase potentiates ligand-dependent proteasome dysfunction induced by polyglutamine-expanded androgen receptor. Human Molecular Genetics. 2003;12(13):1497-506.

Mann DM, Oliver R, Snowden JS. The topographic distribution of brain atrophy in Huntington's disease and progressive supranuclear palsy. Acta Neuropathol. 1993;85(5):553-9.

Manohar S, Paolone NA, Bleichfeld M, Hayes SH, Salvi RJ, Baizer JS. Expression of doublecortin, a neuronal migration protein, in unipolar brush cells of the vestibulocerebellum and dorsal cochlear nucleus of the adult rat. Neuroscience. 2012;202:169-83.

Mao Y, Senic-Matuglia F, Di Fiore PP, Polo S, Hodsdon ME, De Camilli P. Deubiquitinating function of ataxin-3: insights from the solution structure of the Josephin domain. Proc Natl Acad Sci U S A. 2005;102(36):12700-5.

Margolis RL. The spinocerebellar ataxias: order emerges from chaos. Curr Neurol Neurosci Rep. 2002;2(5):447-56.

Martel JC, Alagar R, Robitaille Y, Quirion R. Neuropeptide Y receptor binding sites in human brain. Possible alteration in Alzheimer's disease. Brain Res. 1990;519(1-2):228-35.

Martignoni E, Blandini F, Petraglia F, Pacchetti C, Bono G, Nappi G. Cerebrospinal fluid norepinephrine, 3-methoxy-4-hydroxyphenylglycol and neuropeptide Y levels in Parkinson's disease, multiple system atrophy and dementia of the Alzheimer type. J Neural Transm Park Dis Dement Sect. 1992;4(3):191-205.

Masino L, Musi V, Menon RP, Fusi P, Kelly G, Frenkiel TA, et al. Domain architecture of the polyglutamine protein ataxin-3: a globular domain followed by a flexible tail. FEBS Lett. 2003;549(1-3):21-5.

Masugi F, Ogihara T, Sakaguchi K, Otsuka A, Tsuchiya Y, Morimoto S, et al. High plasma levels of cortisol in patients with senile dementia of the Alzheimer's type. Methods Find Exp Clin Pharmacol. 1989;11(11):707-10.

Matsumoto M, Nomura T, Momose K, Ikeda Y, Kondou Y, Akiho H, et al. Inactivation of a novel neuropeptide Y/peptide YY receptor gene in primate species. J Biol Chem. 1996;271(44):27217-20.

Matsuyama Z, Yanagisawa NK, Aoki Y, Black JL, 3rd, Lennon VA, Mori Y, et al. Polyglutamine repeats of spinocerebellar ataxia 6 impair the cell-death-preventing effect of CaV2.1 Ca2+ channel--loss-of-function cellular model of SCA6. Neurobiol Dis. 2004;17(2):198-204.

Mattson MP, Cheng B, Davis D, Bryant K, Lieberburg I, Rydel RE. beta-Amyloid peptides destabilize calcium homeostasis and render human cortical neurons vulnerable to excitotoxicity. J Neurosci. 1992;12(2):376-89.

Mayford M, Bach ME, Huang YY, Wang L, Hawkins RD, Kandel ER. Control of memory formation through regulated expression of a CaMKII transgene. Science. 1996;274(5293):1678-83.

Mazurek MF, Garside S, Beal MF. Cortical peptide changes in Huntington's disease may be independent of striatal degeneration. Ann Neurol. 1997;41(4):540-7.

McCampbell A, Taylor JP, Taye AA, Robitschek J, Li M, Walcott J, et al. CREB-binding protein sequestration by expanded polyglutamine. Hum Mol Genet. 2000;9(14):2197-202.

McDonald AJ. Coexistence of somatostatin with neuropeptide Y, but not with cholecystokinin or vasoactive intestinal peptide, in neurons of the rat amygdala. Brain Res. 1989;500(1-2):37-45.

McDonald AJ, Mascagni F, Augustine JR. Neuropeptide Y and somatostatin-like immunoreactivity in neurons of the monkey amygdala. Neuroscience. 1995;66(4):959-82.

McDonald AJ, Pearson JC. Coexistence of GABA and peptide immunoreactivity in non-pyramidal neurons of the basolateral amygdala. Neurosci Lett. 1989;100(1-3):53-8.

McDonald WM, Richard IH, DeLong MR. Prevalence, etiology, and treatment of depression in Parkinson's disease. Biol Psychiatry. 2003;54(3):363-75.

McGeer PL, McGeer EG. NSAIDs and Alzheimer disease: epidemiological, animal model and clinical studies. Neurobiol Aging. 2007;28(5):639-47.

McKhann G, Drachman D, Folstein M, Katzman R, Price D, Stadlan EM. Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease. Neurology. 1984;34(7):939-44.

McNaught KS, Belizaire R, Isacson O, Jenner P, Olanow CW. Altered proteasomal function in sporadic Parkinson's disease. Exp Neurol. 2003;179(1):38-46.

McNaught KS, Belizaire R, Jenner P, Olanow CW, Isacson O. Selective loss of 20S proteasome alpha-subunits in the substantia nigra pars compacta in Parkinson's disease. Neurosci Lett. 2002;326(3):155-8.

McNaught KS, Jenner P. Proteasomal function is impaired in substantia nigra in Parkinson's disease. Neurosci Lett. 2001;297(3):191-4.

Medeiros MD, Turner AJ. Processing and metabolism of peptide-YY: pivotal roles of dipeptidylpeptidase-IV, aminopeptidase-P, and endopeptidase-24.11. Endocrinology. 1994;134(5):2088-94.

Medeiros Mdos S, Turner AJ. Metabolism and functions of neuropeptide Y. Neurochem Res. 1996;21(9):1125-32.

Mellios N, Huang HS, Grigorenko A, Rogaev E, Akbarian S. A set of differentially expressed miRNAs, including miR-30a-5p, act as post-transcriptional inhibitors of BDNF in prefrontal cortex. Hum Mol Genet. 2008;17(19):3030-42.

Mendonca LS, Nobrega C, Hirai H, Kaspar BK, Pereira de Almeida L. Transplantation of cerebellar neural stem cells improves motor coordination and neuropathology in Machado-Joseph disease mice. Brain. 2015;138(Pt 2):320-35.

Menzies FM, Huebener J, Renna M, Bonin M, Riess O, Rubinsztein DC. Autophagy induction reduces mutant ataxin-3 levels and toxicity in a mouse model of spinocerebellar ataxia type 3. Brain. 2010;133(Pt 1):93-104.

Mercer RE, Chee MJ, Colmers WF. The role of NPY in hypothalamic mediated food intake. Front Neuroendocrinol. 2011;32(4):398-415.

Michel MC, Beck-Sickinger A, Cox H, Doods HN, Herzog H, Larhammar D, et al. XVI. International Union of Pharmacology recommendations for the nomenclature of

neuropeptide Y, peptide YY, and pancreatic polypeptide receptors. Pharmacol Rev. 1998;50(1):143-50.

Mignot E, Lammers GJ, Ripley B, Okun M, Nevsimalova S, Overeem S, et al. The role of cerebrospinal fluid hypocretin measurement in the diagnosis of narcolepsy and other hypersomnias. Arch Neurol. 2002;59(10):1553-62.

Miguel-Hidalgo JJ, Alvarez XA, Cacabelos R, Quack G. Neuroprotection by memantine against neurodegeneration induced by beta-amyloid(1-40). Brain Res. 2002;958(1):210-21.

Ming GL, Song H. Adult neurogenesis in the mammalian central nervous system. Annu Rev Neurosci. 2005;28:223-50.

Minor RK, Chang JW, de Cabo R. Hungry for life: How the arcuate nucleus and neuropeptide Y may play a critical role in mediating the benefits of calorie restriction. Mol Cell Endocrinol. 2009;299(1):79-88.

Montie HL, Cho MS, Holder L, Liu Y, Tsvetkov AS, Finkbeiner S, et al. Cytoplasmic retention of polyglutamine-expanded androgen receptor ameliorates disease via autophagy in a mouse model of spinal and bulbar muscular atrophy. Hum Mol Genet. 2009;18(11):1937-50.

Mori H, Kondo J, Ihara Y. Ubiquitin is a component of paired helical filaments in Alzheimer's disease. Science. 1987;235(4796):1641-4.

Morrison JH, Hof PR. Life and death of neurons in the aging brain. Science. 1997;278(5337):412-9.

Muchowski PJ, Schaffar G, Sittler A, Wanker EE, Hayer-Hartl MK, Hartl FU. Hsp70 and hsp40 chaperones can inhibit self-assembly of polyglutamine proteins into amyloid-like fibrils. Proc Natl Acad Sci U S A. 2000;97(14):7841-6.

Myers RH, Sax DS, Koroshetz WJ, Mastromauro C, Cupples LA, Kiely DK, et al. Factors associated with slow progression in Huntington's disease. Arch Neurol. 1991;48(8):800-4.

Nagai Y, Inui T, Popiel HA, Fujikake N, Hasegawa K, Urade Y, et al. A toxic monomeric conformer of the polyglutamine protein. Nat Struct Mol Biol. 2007;14(4):332-40.

Nair A, Bonneau RH. Stress-induced elevation of glucocorticoids increases microglia proliferation through NMDA receptor activation. J Neuroimmunol. 2006;171(1-2):72-85.

Nakamura K, Jeong SY, Uchihara T, Anno M, Nagashima K, Nagashima T, et al. SCA17, a novel autosomal dominant cerebellar ataxia caused by an expanded polyglutamine in TATA-binding protein. Hum Mol Genet. 2001;10(14):1441-8.

Nakano KK, Dawson DM, Spence A. Machado disease. A hereditary ataxia in Portuguese emigrants to Massachusetts. Neurology. 1972;22(1):49-55.

Nandagopal R. Dramatic levodopa responsiveness of dystonia in a sporadic case of spinocerebellar ataxia type 3. Postgraduate Medical Journal. 2004;80(944):363-5.

Nascimento-Ferreira I, Nobrega C, Vasconcelos-Ferreira A, Onofre I, Albuquerque D, Aveleira C, et al. Beclin 1 mitigates motor and neuropathological deficits in genetic mouse models of Machado-Joseph disease. Brain. 2013;136(Pt 7):2173-88.

Nascimento-Ferreira I, Santos-Ferreira T, Sousa-Ferreira L, Auregan G, Onofre I, Alves S, et al. Overexpression of the autophagic beclin-1 protein clears mutant ataxin-3 and alleviates Machado-Joseph disease. Brain. 2011;134(Pt 5):1400-15.

Naveilhan P, Neveu I, Arenas E, Ernfors P. Complementary and overlapping expression of Y1, Y2 and Y5 receptors in the developing and adult mouse nervous system. Neuroscience. 1998;87(1):289-302.

Nawa H, Bessho Y, Carnahan J, Nakanishi S, Mizuno K. Regulation of neuropeptide expression in cultured cerebral cortical neurons by brain-derived neurotrophic factor. J Neurochem. 1993;60(2):772-5.

NCT00748956. Dennis Charney, Mount Sinai School of Medicine. Intranasal Administration of Neuropeptide Y in Healthy Male Volunteers (NPY). In: ClinicalTrials.gov [Internet] Cited 2015 March 26. Available from: https://clinicaltrials.gov/ct2/show/NCT00748956 NLM identifier: NCT00748956.

NCT01533519. James Murrough, Mount Sinai School of Medicine. A Dose Escalation Study of Intranasal Neuropeptide Y in Post Traumatic Stress Disorder (PTSD). In: ClnicalTrials.gov [Internet] cited 2005 March 12. Available from: https://clinicaltrials.gov/ct2/show/NCT01533519. NLM Identifier: NCT01533519.

Neveu I, Remy S, Naveilhan P. The neuropeptide Y receptors, Y1 and Y2, are transiently and differentially expressed in the developing cerebellum. Neuroscience. 2002;113(4):767-77.

Nichol KA, Morey A, Couzens MH, Shine J, Herzog H, Cunningham AM. Conservation of expression of neuropeptide Y5 receptor between human and rat hypothalamus and limbic regions suggests an integral role in central neuroendocrine control. J Neurosci. 1999;19(23):10295-304.

Nilsson CL, Brinkmalm A, Minthon L, Blennow K, Ekman R. Processing of neuropeptide Y, galanin, and somatostatin in the cerebrospinal fluid of patients with Alzheimer's disease and frontotemporal dementia. Peptides. 2001;22(12):2105-12.

Nishino S, Ripley B, Overeem S, Lammers GJ, Mignot E. Hypocretin (orexin) deficiency in human narcolepsy. The Lancet. 2000;355(9197):39-40.

Nobre RJ, Almeida LP. Gene therapy for Parkinson's and Alzheimer's diseases: from the bench to clinical trials. Curr Pharm Des. 2011;17(31):3434-45.

Nobrega C, Nascimento-Ferreira I, Onofre I, Albuquerque D, Conceicao M, Deglon N, et al. Overexpression of mutant ataxin-3 in mouse cerebellum induces ataxia and cerebellar neuropathology. Cerebellum. 2013a;12(4):441-55.

Nobrega C, Nascimento-Ferreira I, Onofre I, Albuquerque D, Deglon N, de Almeida LP. RNA interference mitigates motor and neuropathological deficits in a cerebellar mouse model of Machado-Joseph disease. PLoS One. 2014;9(8):e100086.

Nobrega C, Nascimento-Ferreira I, Onofre I, Albuquerque D, Hirai H, Deglon N, et al. Silencing mutant ataxin-3 rescues motor deficits and neuropathology in Machado-Joseph disease transgenic mice. PLoS One. 2013b;8(1):e52396.

Nóbrega C, Pereira de Almeida L. Machado-Joseph Disease / Spinocerebellar Ataxia Type 3. In: Gazulla DJ, editor. Spinocerebellar Ataxia: InTech 2012. p. 103-38.

Noe F, Vaghi V, Balducci C, Fitzsimons H, Bland R, Zardoni D, et al. Anticonvulsant effects and behavioural outcomes of rAAV serotype 1 vector-mediated neuropeptide Y overexpression in rat hippocampus. Gene Ther. 2010;17(5):643-52.

Noe FM, Sorensen AT, Kokaia M, Vezzani A. Gene therapy of focal onset epilepsy using adeno-associated virus vector-mediated overexpression of neuropeptide Y. In: Noebels JL, Avoli M, Rogawski MA, Olsen RW, Delgado-Escueta AV, editors. Jasper's Basic Mechanisms of the Epilepsies. 4th ed. Bethesda (MD); 2012.

O'Sullivan SS, Williams DR, Gallagher DA, Massey LA, Silveira-Moriyama L, Lees AJ. Nonmotor symptoms as presenting complaints in Parkinson's disease: a clinicopathological study. Mov Disord. 2008;23(1):101-6.

Oberto A, Acquadro E, Bus T, Sprengel R, Eva C. Expression patterns of promoters for NPY Y(1) and Y(5) receptors in Y(5)RitTA and Y(1)RVenus BAC-transgenic mice. Eur J Neurosci. 2007;26(1):155-70.

Obuchowicz E, Antkiewicz-Michaluk L, Romanska I, Herman ZS. Increased striatal neuropeptide Y immunoreactivity and its modulation by deprenyl, clonidine and L-dopa in MPTP-treated mice. J Neural Transm. 2003;110(12):1375-91.

Obuchowicz E, Krysiak R, Herman ZS. Does neuropeptide Y (NPY) mediate the effects of psychotropic drugs? Neurosci Biobehav Rev. 2004;28(6):595-610.

Oliveira JM, Chen S, Almeida S, Riley R, Goncalves J, Oliveira CR, et al. Mitochondrial-dependent Ca2+ handling in Huntington's disease striatal cells: effect of histone deacetylase inhibitors. J Neurosci. 2006;26(43):11174-86.

Olney JW. Neurotoxicity of excitatory amino acids. In: McGeer EG, Olney, J. W. and McGeer, P. L., editor. Kainic Acid as a Tool in Neurobiology. New York: Raven Press; 1978. p. 95–112.

Ondo WG, Ben-Aire L, Jankovic J, Lai E, Contant C, Grossman R. Weight gain following unilateral pallidotomy in Parkinson's disease. Acta Neurol Scand. 2000;101(2):79-84.

Ordway JM, Tallaksen-Greene S, Gutekunst CA, Bernstein EM, Cearley JA, Wiener HW, et al. Ectopically expressed CAG repeats cause intranuclear inclusions and a progressive late onset neurological phenotype in the mouse. Cell. 1997;91(6):753-63.

Orr HT, Zoghbi HY. Trinucleotide repeat disorders. Annu Rev Neurosci. 2007;30:575-621.

Ouchi Y, Yoshikawa E, Sekine Y, Futatsubashi M, Kanno T, Ogusu T, et al. Microglial activation and dopamine terminal loss in early Parkinson's disease. Ann Neurol. 2005;57(2):168-75.

Ownby RL, Crocco E, Acevedo A, John V, Loewenstein D. Depression and risk for Alzheimer disease: systematic review, meta-analysis, and metaregression analysis. Arch Gen Psychiatry. 2006;63(5):530-8.

Painsipp E, Herzog H, Holzer P. Implication of neuropeptide-Y Y2 receptors in the effects of immune stress on emotional, locomotor and social behavior of mice. Neuropharmacology. 2008a;55(1):117-26.

Painsipp E, Wultsch T, Edelsbrunner ME, Tasan RO, Singewald N, Herzog H, et al. Reduced anxiety-like and depression-related behavior in neuropeptide Y Y4 receptor knockout mice. Genes Brain Behav. 2008b;7(5):532-42.

Palfi S, Brouillet E, Jarraya B, Bloch J, Jan C, Shin M, et al. Expression of mutated huntingtin fragment in the putamen is sufficient to produce abnormal movement in non-human primates. Mol Ther. 2007;15(8):1444-51.

Pan T, Kondo S, Zhu W, Xie W, Jankovic J, Le W. Neuroprotection of rapamycin in lactacystin-induced neurodegeneration via autophagy enhancement. Neurobiol Dis. 2008;32(1):16-25.

Panov AV, Gutekunst CA, Leavitt BR, Hayden MR, Burke JR, Strittmatter WJ, et al. Early mitochondrial calcium defects in Huntington's disease are a direct effect of polyglutamines. Nat Neurosci. 2002;5(8):731-6.

Park Y, Hong S, Kim SJ, Kang S. Proteasome function is inhibited by polyglutamine-expanded ataxin-1, the SCA1 gene product. Mol Cells. 2005;19(1):23-30.

Paulson HL. Dominantly inherited ataxias: lessons learned from Machado-Joseph disease/spinocerebellar ataxia type 3. Semin Neurol. 2007;27(2):133-42.

Paulson HL, Bonini NM, Roth KA. Polyglutamine disease and neuronal cell death. Proc Natl Acad Sci U S A. 2000;97(24):12957-8.

Paulson HL, Das SS, Crino PB, Perez MK, Patel SC, Gotsdiner D, et al. Machado-Joseph disease gene product is a cytoplasmic protein widely expressed in brain. Ann Neurol. 1997a;41(4):453-62.

Paulson HL, Perez MK, Trottier Y, Trojanowski JQ, Subramony SH, Das SS, et al. Intranuclear inclusions of expanded polyglutamine protein in spinocerebellar ataxia type 3. Neuron. 1997b;19(2):333-44.

Pavese N, Gerhard A, Tai YF, Ho AK, Turkheimer F, Barker RA, et al. Microglial activation correlates with severity in Huntington disease: a clinical and PET study. Neurology. 2006;66(11):1638-43.

Paxinos G, Franklin KBJ, Franklin KBJ. The mouse brain in stereotaxic coordinates. 2nd ed. San Diego: Academic Press; 2001.

Pedrazzini T, Seydoux J, Kunstner P, Aubert JF, Grouzmann E, Beermann F, et al. Cardiovascular response, feeding behavior and locomotor activity in mice lacking the NPY Y1 receptor. Nat Med. 1998;4(6):722-6.

Pedroso JL, Braga-Neto P, Felicio AC, Dutra LA, Santos WA, do Prado GF, et al. Sleep disorders in machado-joseph disease: frequency, discriminative thresholds, predictive values, and correlation with ataxia-related motor and non-motor features. Cerebellum. 2011;10(2):291-5.

Perry G, Friedman R, Shaw G, Chau V. Ubiquitin is detected in neurofibrillary tangles and senile plaque neurites of Alzheimer disease brains. Proc Natl Acad Sci U S A. 1987;84(9):3033-6.

Petersen A, Gil J, Maat-Schieman ML, Bjorkqvist M, Tanila H, Araujo IM, et al. Orexin loss in Huntington's disease. Hum Mol Genet. 2005;14(1):39-47.

Peyron C, Faraco J, Rogers W, Ripley B, Overeem S, Charnay Y, et al. A mutation in a case of early onset narcolepsy and a generalized absence of hypocretin peptides in human narcoleptic brains. Nat Med. 2000;6(9):991-7.

Pfeiffer RF. Gastrointestinal dysfunction in Parkinson's disease. Lancet Neurol. 2003;2(2):107-16.

Pickford F, Masliah E, Britschgi M, Lucin K, Narasimhan R, Jaeger PA, et al. The autophagy-related protein beclin 1 shows reduced expression in early Alzheimer disease and regulates amyloid beta accumulation in mice. J Clin Invest. 2008;118(6):2190-9.

Pike JL, Irwin MR. Dissociation of inflammatory markers and natural killer cell activity in major depressive disorder. Brain Behav Immun. 2006;20(2):169-74.

Politis M, Pavese N, Tai YF, Kiferle L, Mason SL, Brooks DJ, et al. Microglial activation in regions related to cognitive function predicts disease onset in Huntington's disease: a multimodal imaging study. Hum Brain Mapp. 2011;32(2):258-70.

Politis M, Pavese N, Tai YF, Tabrizi SJ, Barker RA, Piccini P. Hypothalamic involvement in Huntington's disease: an in vivo PET study. Brain. 2008a;131(Pt 11):2860-9.

Politis M, Piccini P, Pavese N, Koh SB, Brooks DJ. Evidence of dopamine dysfunction in the hypothalamus of patients with Parkinson's disease: an in vivo 11C-raclopride PET study. Exp Neurol. 2008b;214(1):112-6.

Proto C, Romualdi D, Cento RM, Spada RS, Di Mento G, Ferri R, et al. Plasma levels of neuropeptides in Alzheimer's disease. Gynecol Endocrinol. 2006;22(4):213-8.

Puranam KL, Wu G, Strittmatter WJ, Burke JR. Polyglutamine expansion inhibits respiration by increasing reactive oxygen species in isolated mitochondria. Biochem Biophys Res Commun. 2006;341(2):607-13.

Qian J, Colmers WF, Saggau P. Inhibition of synaptic transmission by neuropeptide Y in rat hippocampal area CA1: modulation of presynaptic Ca2+ entry. J Neurosci. 1997;17(21):8169-77.

Qin L, Liu Y, Cooper C, Liu B, Wilson B, Hong JS. Microglia enhance beta-amyloid peptide-induced toxicity in cortical and mesencephalic neurons by producing reactive oxygen species. J Neurochem. 2002;83(4):973-83.

Qiu C, Kivipelto M, von Strauss E. Epidemiology of Alzheimer's disease: occurrence, determinants, and strategies toward intervention. Dialogues Clin Neurosci. 2009;11(2):111-28.

Qu D, Ludwig DS, Gammeltoft S, Piper M, Pelleymounter MA, Cullen MJ, et al. A role for melanin-concentrating hormone in the central regulation of feeding behaviour. Nature. 1996;380(6571):243-7.

Raadsheer FC, van Heerikhuize JJ, Lucassen PJ, Hoogendijk WJ, Tilders FJ, Swaab DF. Corticotropin-releasing hormone mRNA levels in the paraventricular nucleus of patients with Alzheimer's disease and depression. Am J Psychiatry. 1995;152(9):1372-6.

Rabey JM, Nissipeanu P, Korczyn AD. Efficacy of memantine, an NMDA receptor antagonist, in the treatment of Parkinson's disease. J Neural Transm Park Dis Dement Sect. 1992;4:277-82.

Ramamoorthy P, Whim MD. Trafficking and fusion of neuropeptide Y-containing dense-core granules in astrocytes. J Neurosci. 2008;28(51):13815-27.

Ramos B, Baglietto-Vargas D, del Rio JC, Moreno-Gonzalez I, Santa-Maria C, Jimenez S, et al. Early neuropathology of somatostatin/NPY GABAergic cells in the hippocampus of a PS1xAPP transgenic model of Alzheimer's disease. Neurobiol Aging. 2006;27(11):1658-72.

Ranganathan S, Harmison GG, Meyertholen K, Pennuto M, Burnett BG, Fischbeck KH. Mitochondrial abnormalities in spinal and bulbar muscular atrophy. Hum Mol Genet. 2009;18(1):27-42.

Ranum LP, Lundgren JK, Schut LJ, Ahrens MJ, Perlman S, Aita J, et al. Spinocerebellar ataxia type 1 and Machado-Joseph disease: incidence of CAG expansions among adult-onset ataxia patients from 311 families with dominant, recessive, or sporadic ataxia. Am J Hum Genet. 1995;57(3):603-8.

Raposo M, Vasconcelos J, Bettencourt C, Kay T, Coutinho P, Lima M. Nystagmus as an early ocular alteration in Machado-Joseph disease (MJD/SCA3). BMC Neurol. 2014;14:17.

Ravikumar B, Vacher C, Berger Z, Davies JE, Luo S, Oroz LG, et al. Inhibition of mTOR induces autophagy and reduces toxicity of polyglutamine expansions in fly and mouse models of Huntington disease. Nat Genet. 2004;36(6):585-95.

Redrobe JP, Dumont Y, Fournier A, Baker GB, Quirion R. Role of serotonin (5-HT) in the antidepressant-like properties of neuropeptide Y (NPY) in the mouse forced swim test. Peptides. 2005;26(8):1394-400.

Redrobe JP, Dumont Y, Fournier A, Quirion R. The neuropeptide Y (NPY) Y1 receptor subtype mediates NPY-induced antidepressant-like activity in the mouse forced swimming test. Neuropsychopharmacology. 2002;26(5):615-24.

Reibel S, Vivien-Roels B, Le BT, Larmet Y, Carnahan J, Marescaux C, et al. Overexpression of neuropeptide Y induced by brain-derived neurotrophic factor in the rat hippocampus is long lasting. Eur J Neurosci. 2000;12(2):595-605.

Reinikainen KJ, Soininen H, Riekkinen PJ. Neurotransmitter changes in Alzheimer's disease: implications to diagnostics and therapy. J Neurosci Res. 1990;27(4):576-86.

Remy P, Doder M, Lees A, Turjanski N, Brooks D. Depression in Parkinson's disease: loss of dopamine and noradrenaline innervation in the limbic system. Brain. 2005;128(Pt 6):1314-22.

Richards G, Messer J, Waldvogel HJ, Gibbons HM, Dragunow M, Faull RL, et al. Up-regulation of the isoenzymes MAO-A and MAO-B in the human basal ganglia and pons in Huntington's disease revealed by quantitative enzyme radioautography. Brain Res. 2011;1370:204-14.

Riess O, Rub U, Pastore A, Bauer P, Schols L. SCA3: neurological features, pathogenesis and animal models. Cerebellum. 2008;7(2):125-37.

Rodrigues AJ, Coppola G, Santos C, Costa Mdo C, Ailion M, Sequeiros J, et al. Functional genomics and biochemical characterization of the C. elegans orthologue of the Machado-Joseph disease protein ataxin-3. FASEB J. 2007;21(4):1126-36.

Rodrigues AJ, do Carmo Costa M, Silva TL, Ferreira D, Bajanca F, Logarinho E, et al. Absence of ataxin-3 leads to cytoskeletal disorganization and increased cell death. Biochim Biophys Acta. 2010;1803(10):1154-63.

Rodrigues AJ, Neves-Carvalho A, Teixeira-Castro A, Rokka A, Corthals G, Logarinho E, et al. Absence of ataxin-3 leads to enhanced stress response in C. elegans. PLoS One. 2011;6(4):e18512.

Rodriguez-Lebron E, Costa Mdo C, Luna-Cancalon K, Peron TM, Fischer S, Boudreau RL, et al. Silencing mutant ATXN3 expression resolves molecular phenotypes in SCA3 transgenic mice. Mol Ther. 2013;21(10):1909-18.

Roeske S, Filla I, Heim S, Amunts K, Helmstaedter C, Wullner U, et al. Progressive cognitive dysfunction in spinocerebellar ataxia type 3. Mov Disord. 2013;28(10):1435-8.

Romanul FC, Fowler HL, Radvany J, Feldman RG, Feingold M. Azorean disease of the nervous system. N Engl J Med. 1977;296(26):1505-8.

Rosenberg RN. Machado-Joseph disease: an autosomal dominant motor system degeneration. Mov Disord. 1992;7(3):193-203.

Rosenberg RN. Autosomal dominant cerebellar phenotypes: the genotype has settled the issue. Neurology. 1995;45(1):1-5.

Rosenberg RN, Nyhan WL, Bay C, Shore P. Autosomal dominant striatonigral degeneration. A clinical, pathologic, and biochemical study of a new genetic disorder. Neurology. 1976;26(8):703-14.

Ruano L, Melo C, Silva MC, Coutinho P. The global epidemiology of hereditary ataxia and spastic paraplegia: a systematic review of prevalence studies. Neuroepidemiology. 2014;42(3):174-83.

Rub U, Brunt ER, Petrasch-Parwez E, Schols L, Theegarten D, Auburger G, et al. Degeneration of ingestion-related brainstem nuclei in spinocerebellar ataxia type 2, 3, 6 and 7. Neuropathol Appl Neurobiol. 2006a;32(6):635-49.

Rub U, de Vos RA, Brunt ER, Sebesteny T, Schols L, Auburger G, et al. Spinocerebellar ataxia type 3 (SCA3): thalamic neurodegeneration occurs independently from thalamic ataxin-3 immunopositive neuronal intranuclear inclusions. Brain Pathol. 2006b;16(3):218-27.

Rub U, Del Turco D, Del Tredici K, de Vos RA, Brunt ER, Reifenberger G, et al. Thalamic involvement in a spinocerebellar ataxia type 2 (SCA2) and a spinocerebellar ataxia type 3 (SCA3) patient, and its clinical relevance. Brain. 2003;126(Pt 10):2257-72.

Rub U, Gierga K, Brunt ER, de Vos RA, Bauer M, Schols L, et al. Spinocerebellar ataxias types 2 and 3: degeneration of the pre-cerebellar nuclei isolates the three phylogenetically defined regions of the cerebellum. J Neural Transm. 2005;112(11):1523-45.

Rubinsztein DC. The roles of intracellular protein-degradation pathways in neurodegeneration. Nature. 2006;443(7113):780-6.

Sainsbury A, Schwarzer C, Couzens M, Fetissov S, Furtinger S, Jenkins A, et al. Important role of hypothalamic Y2 receptors in body weight regulation revealed in conditional knockout mice. Proc Natl Acad Sci U S A. 2002a;99(13):8938-43.

Sainsbury A, Schwarzer C, Couzens M, Jenkins A, Oakes SR, Ormandy CJ, et al. Y4 receptor knockout rescues fertility in ob/ob mice. Genes Dev. 2002b;16(9):1077-88.

Sakurai T, Amemiya A, Ishii M, Matsuzaki I, Chemelli RM, Tanaka H, et al. Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. Cell. 1998;92(4):573-85.

Sanberg PR, Fibiger HC, Mark RF. Body weight and dietary factors in Huntington's disease patients compared with matched controls. Med J Aust. 1981;1(8):407-9.

Santos-Carvalho A, Elvas F, Alvaro AR, Ambrosio AF, Cavadas C. Neuropeptide Y receptors activation protects rat retinal neural cells against necrotic and apoptotic cell death induced by glutamate. Cell Death Dis. 2013;4:e636.

Saute JA, da Silva AC, Muller AP, Hansel G, de Mello AS, Maeda F, et al. Serum insulin-like system alterations in patients with spinocerebellar ataxia type 3. Mov Disord. 2011;26(4):731-5.

Saute JA, Silva AC, Souza GN, Russo AD, Donis KC, Vedolin L, et al. Body mass index is inversely correlated with the expanded CAG repeat length in SCA3/MJD patients. Cerebellum. 2012;11(3):771-4.

Scheel H, Tomiuk S, Hofmann K. Elucidation of ataxin-3 and ataxin-7 function by integrative bioinformatics. Hum Mol Genet. 2003;12(21):2845-52.

Schmidt T, Landwehrmeyer GB, Schmitt I, Trottier Y, Auburger G, Laccone F, et al. An isoform of ataxin-3 accumulates in the nucleus of neuronal cells in affected brain regions of SCA3 patients. Brain Pathol. 1998;8(4):669-79.

Schmidt T, Lindenberg KS, Krebs A, Schols L, Laccone F, Herms J, et al. Protein surveillance machinery in brains with spinocerebellar ataxia type 3: redistribution and differential recruitment of 26S proteasome subunits and chaperones to neuronal intranuclear inclusions. Ann Neurol. 2002;51(3):302-10.

Schmitt I, Linden M, Khazneh H, Evert BO, Breuer P, Klockgether T, et al. Inactivation of the mouse Atxn3 (ataxin-3) gene increases protein ubiquitination. Biochem Biophys Res Commun. 2007;362(3):734-9.

Schmitz-Hubsch T, Coudert M, Tezenas du Montcel S, Giunti P, Labrum R, Durr A, et al. Depression comorbidity in spinocerebellar ataxia. Mov Disord. 2011;26(5):870-6.

Schöls L, Bauer P, Schmidt T, Schulte T, Riess O. Autosomal dominant cerebellar ataxias: clinical features, genetics, and pathogenesis. The Lancet Neurology. 2004;3(5):291-304.

Schols L, Haan J, Riess O, Amoiridis G, Przuntek H. Sleep disturbance in spinocerebellar ataxias: Is the SCA3 mutation a cause of restless legs syndrome? Neurology. 1998;51(6):1603-7.

Schultzberg M, Lindberg C, Aronsson AF, Hjorth E, Spulber SD, Oprica M. Inflammation in the nervous system--physiological and pathophysiological aspects. Physiol Behav. 2007;92(1-2):121-8.

Schwartzberg M, Unger J, Weindl A, Lange W. Distribution of neuropeptide Y in the prosencephalon of man and cotton-head tamarin (Saguinus oedipus): colocalization with somatostatin in neurons of striatum and amygdala. Anat Embryol (Berl). 1990;181(2):157-66.

Schwarzer C, Sperk G, Samanin R, Rizzi M, Gariboldi M, Vezzani A. Neuropeptides-immunoreactivity and their mRNA expression in kindling: functional implications for limbic epileptogenesis. Brain Res Brain Res Rev. 1996;22(1):27-50.

Senut MC, Suhr ST, Kaspar B, Gage FH. Intraneuronal aggregate formation and cell death after viral expression of expanded polyglutamine tracts in the adult rat brain. J Neurosci. 2000;20(1):219-29.

Seo H, Sonntag KC, Isacson O. Generalized brain and skin proteasome inhibition in Huntington's disease. Ann Neurol. 2004;56(3):319-28.

Sequeiros J, Coutinho P. Epidemiology and clinical aspects of Machado-Joseph disease. Adv Neurol. 1993;61:139-53.

Serova LI, Tillinger A, Alaluf LG, Laukova M, Keegan K, Sabban EL. Single intranasal neuropeptide Y infusion attenuates development of PTSD-like symptoms to traumatic stress in rats. Neuroscience. 2013;236:298-312.

Serra HG, Byam CE, Lande JD, Tousey SK, Zoghbi HY, Orr HT. Gene profiling links SCA1 pathophysiology to glutamate signaling in Purkinje cells of transgenic mice. Hum Mol Genet. 2004;13(20):2535-43.

Shao J, Diamond MI. Polyglutamine diseases: emerging concepts in pathogenesis and therapy. Hum Mol Genet. 2007;16 Spec No. 2:R115-23.

Shehadeh J, Fernandes HB, Zeron Mullins MM, Graham RK, Leavitt BR, Hayden MR, et al. Striatal neuronal apoptosis is preferentially enhanced by NMDA receptor activation in YAC transgenic mouse model of Huntington disease. Neurobiol Dis. 2006;21(2):392-403.

Sheriff S, Chance WT, Fischer JE, Balasubramaniam A. Neuropeptide Y treatment and food deprivation increase cyclic AMP response element-binding in rat hypothalamus. Mol Pharmacol. 1997;51(4):597-604.

Shevtsova Z, Malik JM, Michel U, Bahr M, Kugler S. Promoters and serotypes: targeting of adeno-associated virus vectors for gene transfer in the rat central nervous system in vitro and in vivo. Exp Physiol. 2005;90(1):53-9.

Shi Y, Wang C, Huang F, Chen Z, Sun Z, Wang J, et al. High Serum GFAP Levels in SCA3/MJD May Not Correlate with Disease Progression. Cerebellum. 2015.

Shibata M, Lu T, Furuya T, Degterev A, Mizushima N, Yoshimori T, et al. Regulation of intracellular accumulation of mutant Huntingtin by Beclin 1. J Biol Chem. 2006;281(20):14474-85.

Shimada M, Tritos NA, Lowell BB, Flier JS, Maratos-Flier E. Mice lacking melanin-concentrating hormone are hypophagic and lean. Nature. 1998;396(6712):670-4.

Shirbin CA, Chua P, Churchyard A, Lowndes G, Hannan AJ, Pang TY, et al. Cortisol and depression in pre-diagnosed and early stage Huntington's disease. Psychoneuroendocrinology. 2013;38(11):2439-47.

Silva-Fernandes A, Costa Mdo C, Duarte-Silva S, Oliveira P, Botelho CM, Martins L, et al. Motor uncoordination and neuropathology in a transgenic mouse model of Machado-Joseph disease lacking intranuclear inclusions and ataxin-3 cleavage products. Neurobiol Dis. 2010;40(1):163-76.

Silva-Fernandes A, Duarte-Silva S, Neves-Carvalho A, Amorim M, Soares-Cunha C, Oliveira P, et al. Chronic treatment with 17-DMAG improves balance and coordination in a new mouse model of Machado-Joseph disease. Neurotherapeutics. 2014;11(2):433-49.

Silva AP, Pinheiro PS, Carvalho AP, Carvalho CM, Jakobsen B, Zimmer J, et al. Activation of neuropeptide Y receptors is neuroprotective against excitotoxicity in organotypic hippocampal slice cultures. FASEB J. 2003;17(9):1118-20.

Silva AP, Xapelli S, Grouzmann E, Cavadas C. The putative neuroprotective role of neuropeptide Y in the central nervous system. Curr Drug Targets CNS Neurol Disord. 2005;4(4):331-47.

Simoes AT, Goncalves N, Koeppen A, Deglon N, Kugler S, Duarte CB, et al. Calpastatin-mediated inhibition of calpains in the mouse brain prevents mutant ataxin 3 proteolysis, nuclear localization and aggregation, relieving Machado-Joseph disease. Brain. 2012;135(Pt 8):2428-39.

Simoes AT, Goncalves N, Nobre RJ, Duarte CB, Pereira de Almeida L. Calpain inhibition reduces ataxin-3 cleavage alleviating neuropathology and motor impairments in mouse models of Machado-Joseph disease. Hum Mol Genet. 2014;23(18):4932-44.

Skinner PJ, Koshy BT, Cummings CJ, Klement IA, Helin K, Servadio A, et al. Ataxin-1 with an expanded glutamine tract alters nuclear matrix-associated structures. Nature. 1997;389(6654):971-4.

Smialowska M, Domin H, Zieba B, Kozniewska E, Michalik R, Piotrowski P, et al. Neuroprotective effects of neuropeptide Y-Y2 and Y5 receptor agonists in vitro and in vivo. Neuropeptides. 2009;43(3):235-49.

Smith DL, Woodman B, Mahal A, Sathasivam K, Ghazi-Noori S, Lowden PA, et al. Minocycline and doxycycline are not beneficial in a model of Huntington's disease. Ann Neurol. 2003;54(2):186-96.

Smith Y, Parent A. Neuropeptide Y-immunoreactive neurons in the striatum of cat and monkey: morphological characteristics, intrinsic organization and co-localization with somatostatin. Brain Res. 1986;372(2):241-52.

Soneson C, Fontes M, Zhou Y, Denisov V, Paulsen JS, Kirik D, et al. Early changes in the hypothalamic region in prodromal Huntington disease revealed by MRI analysis. Neurobiol Dis. 2010;40(3):531-43.

Soong BW, Liu RS. Positron emission tomography in asymptomatic gene carriers of Machado-Joseph disease. J Neurol Neurosurg Psychiatry. 1998;64(4):499-504.

Soto ME, Secher M, Gillette-Guyonnet S, Abellan van Kan G, Andrieu S, Nourhashemi F, et al. Weight loss and rapid cognitive decline in community-dwelling patients with Alzheimer's disease. J Alzheimers Dis. 2012;28(3):647-54.

Sousa-Ferreira L, Garrido M, Nascimento-Ferreira I, Nobrega C, Santos-Carvalho A, Alvaro AR, et al. Moderate long-term modulation of neuropeptide Y in hypothalamic arcuate nucleus induces energy balance alterations in adult rats. PLoS One. 2011;6(7):e22333.

Spencer B, Potkar R, Trejo M, Rockenstein E, Patrick C, Gindi R, et al. Beclin 1 gene transfer activates autophagy and ameliorates the neurodegenerative pathology in alpha-synuclein models of Parkinson's and Lewy body diseases. J Neurosci. 2009;29(43):13578-88.

Spilman P, Podlutskaya N, Hart MJ, Debnath J, Gorostiza O, Bredesen D, et al. Inhibition of mTOR by rapamycin abolishes cognitive deficits and reduces amyloid-beta levels in a mouse model of Alzheimer's disease. PLoS One. 2010;5(4):e9979.

Standaert DG, Lee VM, Greenberg BD, Lowery DE, Trojanowski JQ. Molecular features of hypothalamic plaques in Alzheimer's disease. Am J Pathol. 1991;139(3):681-91.

Stanic D, Brumovsky P, Fetissov S, Shuster S, Herzog H, Hokfelt T. Characterization of neuropeptide Y2 receptor protein expression in the mouse brain. I. Distribution in cell bodies and nerve terminals. J Comp Neurol. 2006;499(3):357-90.

Stanic D, Mulder J, Watanabe M, Hokfelt T. Characterization of NPY Y2 receptor protein expression in the mouse brain. II. Coexistence with NPY, the Y1 receptor, and other neurotransmitter-related molecules. J Comp Neurol. 2011;519(7):1219-57.

Stanley BG, Leibowitz SF. Neuropeptide Y injected in the paraventricular hypothalamus: a powerful stimulant of feeding behavior. Proc Natl Acad Sci U S A. 1985;82(11):3940-3.

Starback P, Wraith A, Eriksson H, Larhammar D. Neuropeptide Y receptor gene y6: multiple deaths or resurrections? Biochem Biophys Res Commun. 2000;277(1):264-9.

Starkstein SE, Jorge R, Mizrahi R, Robinson RG. The construct of minor and major depression in Alzheimer's disease. Am J Psychiatry. 2005;162(11):2086-93.

Steward LJ, Bufton KE, Hopkins PC, Davies WE, Barnes NM. Reduced levels of 5-HT3 receptor recognition sites in the putamen of patients with Huntington's disease. Eur J Pharmacol. 1993;242(2):137-43.

Stogner KA, Holmes PV. Neuropeptide-Y exerts antidepressant-like effects in the forced swim test in rats. Eur J Pharmacol. 2000;387(2):R9-10.

Sudarsky L, Coutinho P. Machado-Joseph disease. Clin Neurosci. 1995;3(1):17-22.

Sun Y, Savanenin A, Reddy PH, Liu YF. Polyglutamine-expanded huntingtin promotes sensitization of N-methyl-D-aspartate receptors via post-synaptic density 95. J Biol Chem. 2001;276(27):24713-8.

Sunderland T, Berrettini WH, Molchan SE, Lawlor BA, Martinez RA, Vitiello B, et al. Reduced cerebrospinal fluid dynorphin A1-8 in Alzheimer's disease. Biol Psychiatry. 1991;30(1):81-7.

Swaab DF. Neuropeptides in hypothalamic neuronal disorders. Int Rev Cytol. 2004;240:305-75.

Swart I, Jahng JW, Overton JM, Houpt TA. Hypothalamic NPY, AGRP, and POMC mRNA responses to leptin and refeeding in mice. Am J Physiol Regul Integr Comp Physiol. 2002;283(5):R1020-6.

Tai YF, Pavese N, Gerhard A, Tabrizi SJ, Barker RA, Brooks DJ, et al. Microglial activation in presymptomatic Huntington's disease gene carriers. Brain. 2007;130(Pt 7):1759-66.

Tait D, Riccio M, Sittler A, Scherzinger E, Santi S, Ognibene A, et al. Ataxin-3 is transported into the nucleus and associates with the nuclear matrix. Hum Mol Genet. 1998;7(6):991-7.

Takahashi M, Ishikawa K, Sato N, Obayashi M, Niimi Y, Ishiguro T, et al. Reduced brain-derived neurotrophic factor (BDNF) mRNA expression and presence of BDNF-immunoreactive granules in the spinocerebellar ataxia type 6 (SCA6) cerebellum. Neuropathology. 2012;32(6):595-603.

Takiyama Y, Nishizawa M, Tanaka H, Kawashima S, Sakamoto H, Karube Y, et al. The gene for Machado-Joseph disease maps to human chromosome 14q. Nat Genet. 1993;4(3):300-4.

Tanaka Y, Engelender S, Igarashi S, Rao RK, Wanner T, Tanzi RE, et al. Inducible expression of mutant alpha-synuclein decreases proteasome activity and increases sensitivity to mitochondria-dependent apoptosis. Hum Mol Genet. 2001;10(9):919-26.

Tang TS, Slow E, Lupu V, Stavrovskaya IG, Sugimori M, Llinas R, et al. Disturbed Ca2+ signaling and apoptosis of medium spiny neurons in Huntington's disease. Proc Natl Acad Sci U S A. 2005;102(7):2602-7.

Taniwaki T, Sakai T, Kobayashi T, Kuwabara Y, Otsuka M, Ichiya Y, et al. Positron emission tomography (PET) in Machado-Joseph disease. J Neurol Sci. 1997;145(1):63-7.

Tao X, Finkbeiner S, Arnold DB, Shaywitz AJ, Greenberg ME. Ca2+ influx regulates BDNF transcription by a CREB family transcription factor-dependent mechanism. Neuron. 1998;20(4):709-26.

Tarlac V, Storey E. Role of proteolysis in polyglutamine disorders. J Neurosci Res. 2003;74(3):406-16.

Taroni F, DiDonato S. Pathways to motor incoordination: the inherited ataxias. Nat Rev Neurosci. 2004;5(8):641-55.

Tasan RO, Lin S, Hetzenauer A, Singewald N, Herzog H, Sperk G. Increased novelty-induced motor activity and reduced depression-like behavior in neuropeptide Y (NPY)-Y4 receptor knockout mice. Neuroscience. 2009;158(4):1717-30.

Tatemoto K. Neuropeptide Y: complete amino acid sequence of the brain peptide. Proc Natl Acad Sci U S A. 1982;79(18):5485-9.

Tatemoto K, Carlquist M, Mutt V. Neuropeptide Y--a novel brain peptide with structural similarities to peptide YY and pancreatic polypeptide. Nature. 1982;296(5858):659-60.

Tehranian R, Hasanvan H, Iverfeldt K, Post C, Schultzberg M. Early induction of interleukin-6 mRNA in the hippocampus and cortex of APPsw transgenic mice Tg2576. Neurosci Lett. 2001;301(1):54-8.

Teixeira-Castro A, Ailion M, Jalles A, Brignull HR, Vilaca JL, Dias N, et al. Neuron-specific proteotoxicity of mutant ataxin-3 in C. elegans: rescue by the DAF-16 and HSF-1 pathways. Hum Mol Genet. 2011;20(15):2996-3009.

Thannickal TC, Lai YY, Siegel JM. Hypocretin (orexin) cell loss in Parkinson's disease. Brain. 2007;130(Pt 6):1586-95.

Thannickal TC, Moore RY, Nienhuis R, Ramanathan L, Gulyani S, Aldrich M, et al. Reduced number of hypocretin neurons in human narcolepsy. Neuron. 2000;27(3):469-74.

The Huntington's Disease Collaborative Research G. A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. Cell. 1993;72(6):971-83.

Thiriet N, Deng X, Solinas M, Ladenheim B, Curtis W, Goldberg SR, et al. Neuropeptide Y protects against methamphetamine-induced neuronal apoptosis in the mouse striatum. J Neurosci. 2005;25(22):5273-9.

Thomas CE, Ehrhardt A, Kay MA. Progress and problems with the use of viral vectors for gene therapy. Nat Rev Genet. 2003;4(5):346-58.

Torashima T, Koyama C, Iizuka A, Mitsumura K, Takayama K, Yanagi S, et al. Lentivector-mediated rescue from cerebellar ataxia in a mouse model of spinocerebellar ataxia. EMBO Rep. 2008;9(4):393-9.

Trejo A, Tarrats RM, Alonso ME, Boll MC, Ochoa A, Velasquez L. Assessment of the nutrition status of patients with Huntington's disease. Nutrition. 2004;20(2):192-6.

Trottier Y, Cancel G, An-Gourfinkel I, Lutz Y, Weber C, Brice A, et al. Heterogeneous intracellular localization and expression of ataxin-3. Neurobiol Dis. 1998;5(5):335-47.

Tsai HF, Tsai HJ, Hsieh M. Full-length expanded ataxin-3 enhances mitochondrial-mediated cell death and decreases Bcl-2 expression in human neuroblastoma cells. Biochem Biophys Res Commun. 2004;324(4):1274-82.

Tsai YC, Fishman PS, Thakor NV, Oyler GA. Parkin facilitates the elimination of expanded polyglutamine proteins and leads to preservation of proteasome function. J Biol Chem. 2003;278(24):22044-55.

Tschenett A, Singewald N, Carli M, Balducci C, Salchner P, Vezzani A, et al. Reduced anxiety and improved stress coping ability in mice lacking NPY-Y2 receptors. Eur J Neurosci. 2003;18(1):143-8.

Tuite PJ, Rogaeva EA, St George-Hyslop PH, Lang AE. Dopa-responsive parkinsonism phenotype of Machado-Joseph disease: confirmation of 14q CAG expansion. Ann Neurol. 1995;38(4):684-7.

Uitti RJ, Rajput AH, Ahlskog JE, Offord KP, Schroeder DR, Ho MM, et al. Amantadine treatment is an independent predictor of improved survival in Parkinson's disease. Neurology. 1996;46(6):1551-6.

Urrea C, Castellanos DA, Sagen J, Tsoulfas P, Bramlett HM, Dietrich WD. Widespread cellular proliferation and focal neurogenesis after traumatic brain injury in the rat. Restor Neurol Neurosci. 2007;25(1):65-76.

Vaarmann A, Kovac S, Holmstrom KM, Gandhi S, Abramov AY. Dopamine protects neurons against glutamate-induced excitotoxicity. Cell Death Dis. 2013;4:e455.

Valassi E, Scacchi M, Cavagnini F. Neuroendocrine control of food intake. Nutr Metab Cardiovasc Dis. 2008;18(2):158-68.

van de Warrenburg BP, Sinke RJ, Verschuuren-Bemelmans CC, Scheffer H, Brunt ER, Ippel PF, et al. Spinocerebellar ataxias in the Netherlands: prevalence and age at onset variance analysis. Neurology. 2002;58(5):702-8.

van der Marck MA, Dicke HC, Uc EY, Kentin ZH, Borm GF, Bloem BR, et al. Body mass index in Parkinson's disease: a meta-analysis. Parkinsonism Relat Disord. 2012;18(3):263-7.

van Duijn E, Selis MA, Giltay EJ, Zitman FG, Roos RA, van Pelt H, et al. Hypothalamic-pituitary-adrenal axis functioning in Huntington's disease mutation carriers compared with mutation-negative first-degree controls. Brain Res Bull. 2010;83(5):232-7.

Vehmas AK, Kawas CH, Stewart WF, Troncoso JC. Immune reactive cells in senile plaques and cognitive decline in Alzheimer's disease. Neurobiol Aging. 2003;24(2):321-31.

Verret L, Goutagny R, Fort P, Cagnon L, Salvert D, Leger L, et al. A role of melanin-concentrating hormone producing neurons in the central regulation of paradoxical sleep. BMC Neurosci. 2003;4:19.

Vezzani A, Schwarzer C, Lothman EW, Williamson J, Sperk G. Functional changes in somatostatin and neuropeptide Y containing neurons in the rat hippocampus in chronic models of limbic seizures. Epilepsy Res. 1996;26(1):267-79.

Vig PJ, Shao Q, Subramony SH, Lopez ME, Safaya E. Bergmann glial S100B activates myo-inositol monophosphatase 1 and Co-localizes to purkinje cell vacuoles in SCA1 transgenic mice. Cerebellum. 2009;8(3):231-44.

Vonsattel JP, DiFiglia M. Huntington disease. J Neuropathol Exp Neurol. 1998;57(5):369-84.

Walker MW, Wolinsky TD, Jubian V, Chandrasena G, Zhong H, Huang X, et al. The novel neuropeptide Y Y5 receptor antagonist Lu AA33810 [N-[[trans-4-[(4,5-dihydro[1]benzothiepino[5,4-d]thiazol-2-yl)amino]cyclohexyl]me thyl]-methanesulfonamide] exerts anxiolytic- and antidepressant-like effects in rat models of stress sensitivity. J Pharmacol Exp Ther. 2009;328(3):900-11.

Wang G, Ide K, Nukina N, Goto J, Ichikawa Y, Uchida K, et al. Machado-Joseph disease gene product identified in lymphocytes and brain. Biochem Biophys Res Commun. 1997;233(2):476-9.

Wang HL, He CY, Chou AH, Yeh TH, Chen YL, Li AH. Polyglutamine-expanded ataxin-7 decreases nuclear translocation of NF-kappaB p65 and impairs NF-kappaB activity by inhibiting proteasome activity of cerebellar neurons. Cell Signal. 2007;19(3):573-81.

Wang HL, Yeh TH, Chou AH, Kuo YL, Luo LJ, He CY, et al. Polyglutamine-expanded ataxin-7 activates mitochondrial apoptotic pathway of cerebellar neurons by upregulating Bax and downregulating Bcl-x(L). Cell Signal. 2006;18(4):541-52.

Wang J, Wang CE, Orr A, Tydlacka S, Li SH, Li XJ. Impaired ubiquitin-proteasome system activity in the synapses of Huntington's disease mice. J Cell Biol. 2008;180(6):1177-89.

Wang PN, Yang CL, Lin KN, Chen WT, Chwang LC, Liu HC. Weight loss, nutritional status and physical activity in patients with Alzheimer's disease. A controlled study. J Neurol. 2004;251(3):314-20.

Warrick JM, Morabito LM, Bilen J, Gordesky-Gold B, Faust LZ, Paulson HL, et al. Ataxin-3 suppresses polyglutamine neurodegeneration in Drosophila by a ubiquitin-associated mechanism. Mol Cell. 2005;18(1):37-48.

Warrick JM, Paulson HL, Gray-Board GL, Bui QT, Fischbeck KH, Pittman RN, et al. Expanded polyglutamine protein forms nuclear inclusions and causes neural degeneration in Drosophila. Cell. 1998;93(6):939-49.

Watanabe Y, Tatebe H, Taguchi K, Endo Y, Tokuda T, Mizuno T, et al. p62/SQSTM1-dependent autophagy of Lewy body-like alpha-synuclein inclusions. PLoS One. 2012;7(12):e52868.

Wee Yong V. Inflammation in neurological disorders: a help or a hindrance? Neuroscientist. 2010;16(4):408-20.

White H, Pieper C, Schmader K. The association of weight change in Alzheimer's disease with severity of disease and mortality: a longitudinal analysis. J Am Geriatr Soc. 1998;46(10):1223-7.

White H, Pieper C, Schmader K, Fillenbaum G. A longitudinal analysis of weight change in Alzheimer's disease. J Am Geriatr Soc. 1997;45(4):531-2.

Williams RH, Morton AJ, Burdakov D. Paradoxical function of orexin/hypocretin circuits in a mouse model of Huntington's disease. Neurobiol Dis. 2011;42(3):438-45.

Willie JT, Sinton CM, Maratos-Flier E, Yanagisawa M. Abnormal response of melanin-concentrating hormone deficient mice to fasting: hyperactivity and rapid eye movement sleep suppression. Neuroscience. 2008;156(4):819-29.

Winner B, Winkler J. Adult neurogenesis in neurodegenerative diseases. Cold Spring Harb Perspect Biol. 2015;7(4):a021287.

Wirth MJ, Patz S, Wahle P. Transcellular induction of neuropeptide Y expression by NT4 and BDNF. Proc Natl Acad Sci U S A. 2005;102(8):3064-9.

Wolak ML, DeJoseph MR, Cator AD, Mokashi AS, Brownfield MS, Urban JH. Comparative distribution of neuropeptide Y Y1 and Y5 receptors in the rat brain by using immunohistochemistry. J Comp Neurol. 2003;464(3):285-311.

Wong E, Cuervo AM. Integration of clearance mechanisms: the proteasome and autophagy. Cold Spring Harb Perspect Biol. 2010;2(12):a006734.

Woods BT, Schaumburg HH. Nigro-spino-dentatal degeneration with nuclear ophthalmoplegia. A unique and partially treatable clinico-pathological entity. J Neurol Sci. 1972;17(2):149-66.

Wu SY, Wang TF, Yu L, Jen CJ, Chuang JI, Wu FS, et al. Running exercise protects the substantia nigra dopaminergic neurons against inflammation-induced degeneration via the activation of BDNF signaling pathway. Brain Behav Immun. 2011;25(1):135-46.

Wu YF, Li SB. Neuropeptide Y expression in mouse hippocampus and its role in neuronal excitotoxicity. Acta Pharmacol Sin. 2005;26(1):63-8.

Wullner U, Reimold M, Abele M, Burk K, Minnerop M, Dohmen BM, et al. Dopamine transporter positron emission tomography in spinocerebellar ataxias type 1, 2, 3, and 6. Arch Neurol. 2005;62(8):1280-5.

Xapelli S, Bernardino L, Ferreira R, Grade S, Silva AP, Salgado JR, et al. Interaction between neuropeptide Y (NPY) and brain-derived neurotrophic factor in NPY-mediated neuroprotection against excitotoxicity: a role for microglia. Eur J Neurosci. 2008;27(8):2089-102.

Xia H, Mao Q, Eliason SL, Harper SQ, Martins IH, Orr HT, et al. RNAi suppresses polyglutamine-induced neurodegeneration in a model of spinocerebellar ataxia. Nat Med. 2004;10(8):816-20.

Xie W, Li X, Li C, Zhu W, Jankovic J, Le W. Proteasome inhibition modeling nigral neuron degeneration in Parkinson's disease. J Neurochem. 2010a;115(1):188-99.

Xie Y, Hayden MR, Xu B. BDNF overexpression in the forebrain rescues Huntington's disease phenotypes in YAC128 mice. J Neurosci. 2010b;30(44):14708-18.

Yaksh TL, Carmichael SW, Stoddard SL, Tyce GM, Kelly PJ, Lucas D, et al. Measurement of lumbar CSF levels of met-enkephalin, encrypted met-enkephalin, and neuropeptide Y in normal patients and in patients with Parkinson's disease before and after autologous transplantation of adrenal medulla into the caudate nucleus. J Lab Clin Med. 1990;115(3):346-51.

Yan Q, Zhang J, Liu H, Babu-Khan S, Vassar R, Biere AL, et al. Anti-inflammatory drug therapy alters beta-amyloid processing and deposition in an animal model of Alzheimer's disease. J Neurosci. 2003;23(20):7504-9.

Yang W, Dunlap JR, Andrews RB, Wetzel R. Aggregated polyglutamine peptides delivered to nuclei are toxic to mammalian cells. Hum Mol Genet. 2002;11(23):2905-17.

Yaron A, Mlynar D. Aminopeptidase-P. Biochem Biophys Res Commun. 1968;32(4):658-63.

Yarosh HL, Angulo JA. Modulation of methamphetamine-induced nitric oxide production by neuropeptide Y in the murine striatum. Brain Res. 2012;1483:31-8.

Yu YC, Kuo CL, Cheng WL, Liu CS, Hsieh M. Decreased antioxidant enzyme activity and increased mitochondrial DNA damage in cellular models of Machado-Joseph disease. J Neurosci Res. 2009;87(8):1884-91.

Zeron MM, Fernandes HB, Krebs C, Shehadeh J, Wellington CL, Leavitt BR, et al. Potentiation of NMDA receptor-mediated excitotoxicity linked with intrinsic apoptotic pathway in YAC transgenic mouse model of Huntington's disease. Mol Cell Neurosci. 2004;25(3):469-79.

Zhang H, Li Q, Graham RK, Slow E, Hayden MR, Bezprozvanny I. Full length mutant huntingtin is required for altered Ca2+ signaling and apoptosis of striatal neurons in the YAC mouse model of Huntington's disease. Neurobiol Dis. 2008;31(1):80-8.

Zhao C, Deng W, Gage FH. Mechanisms and functional implications of adult neurogenesis. Cell. 2008;132(4):645-60.

Zhao M, Momma S, Delfani K, Carlen M, Cassidy RM, Johansson CB, et al. Evidence for neurogenesis in the adult mammalian substantia nigra. Proc Natl Acad Sci U S A. 2003;100(13):7925-30.

Zoghbi HY, Orr HT. Glutamine repeats and neurodegeneration. Annu Rev Neurosci. 2000;23:217-47.