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# Modeling C9ORF72 loss-of-function : a knockdown mouse model

Maria Belen Lopez-Herdoiza

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# Université Pierre et Marie Curie

Ecole Cerveau, Cognition, Comportement (ED-158)

*Molecular Basis and Treatment of Neurodegenerative diseases*

## **Modeling C9ORF72 loss-of-function: a knockdown mouse model**

By Maria-Belen LOPEZ HERDOIZA

Submitted and defended in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in NEUROSCIENCE

on September 27<sup>th</sup>, 2016

In front of a jury composed of:

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*“A good question is, of course, the  
key by which infinite answers can  
be educed”.*

*Asimov (1982)*

*“Let us keep looking, in spite of everything.  
Let us keep searching. It is indeed the best  
method of finding, and perhaps thanks to our  
efforts, the verdict we will give such a patient  
tomorrow will not be the same we must give  
this man today.”*

*Charcot (1889)*

*Para Amira y Carlos,*



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## French abstract – Résumé en français détaillé

Les démences frontotemporales (DFT) et la sclérose latérale amyotrophique (SLA) sont deux maladies neurodégénératives dévastatrices. La mutation du gène *C9ORF72* a été identifiée comme la cause commune la plus fréquente de ces deux pathologies (c9FTD/ALS). Depuis la découverte de cette mutation, de nombreux efforts ont été faits pour identifier les mécanismes pathogéniques et ainsi développer de nouvelles thérapies. La mutation du gène *C9ORF72* correspond à une expansion GGGGCC située dans le premier intron pour les transcrits V1 et V3 ou dans le promoteur du transcrit V2. Chez les patients, l'expansion hexanucléotidique est répétée plusieurs centaines de fois (~700 à 1,000) alors que les individus sains ne présentent pas plus de 30 répétitions. Plusieurs hypothèses pourraient expliquer les mécanismes conférant un effet toxique à la mutation et amenant à une neurodégénérescence. En premier lieu une toxicité de l'ARN mutant peut résulter de l'accumulation d'ARNs contenant les expansions dans des foci nucléaires séquestrant des protéines de liaison à l'ARN et des éléments d'épissage. Ces transcrits avec expansion peuvent aussi être traduits par un mécanisme non conventionnel indépendant de l'ATG (traduction RAN). Les dipeptides répétés résultants de cette traduction s'agrègent et pourraient s'avérer toxiques. Finalement, il a été montré que l'expansion cause la réduction de l'expression du gène *C9ORF72* provoquant ainsi un phénomène d'haploinsuffisance qui pourrait lui aussi être délétère. Ces différentes hypothèses ne sont pas mutuellement exclusives et la neurodégénérescence peut être due à leur association. Il est donc indispensable de différencier les implications individuelles de la toxicité de l'ARN et des dipeptides ainsi que l'effet de l'haploinsuffisance.

Dans ce contexte, nous nous sommes intéressés à l'effet de la perte de fonction du gène *C9ORF72*. Pour étudier sa relation avec la DFT et la SLA, nous avons développé un modèle murin en utilisant un micro ARN interférence de synthèse pour diminuer les transcrits du gène *C9orf72* murin et ainsi obtenir des souris avec un knockdown ubiquitaire de *C9orf72*, appelées souris miR-*C9orf72*. Cette méthode nous a permis d'obtenir des souris exprimant *C9orf72* à un niveau semblable à celui des patients, tout en respectant la même variabilité (20 à 70% d'expression). Nous avons réalisé une caractérisation exhaustive de ces souris afin d'identifier des altérations au niveau comportemental, histologique et cellulaire se rapprochant des atteintes de type DFT et/ou SLA.



Nous avons montré que les souris miR-C9orf72 développent une atteinte de type DFT tout en présentant une motricité normale. A partir de l'âge de 5 mois, les souris miR-C9orf72 présentent une altération du comportement social ainsi qu'un comportement de type dépressif, anomalies typiques que l'on peut retrouver chez les modèles murins de DFT. Cependant, l'activité synaptique hippocampique, qui est l'un des supports majeurs des comportements sociaux chez la souris, semble conservée chez ces souris en vieillissant. Les souris miR-C9orf72 ne présentent pas de troubles majeurs de locomotion ou d'équilibre, ni de perte de motoneurones, ce qui exclut une atteinte de type SLA. Toutefois, nous avons observé un déficit de force musculaire à partir de l'âge de 12 mois malgré une morphologie du tissu musculaire intacte. Ce déficit de force pourrait s'expliquer par une atteinte de la jonction neuromusculaire (JNM). En effet, même si le contact entre les régions pre- et post-synaptiques de la JNM est maintenu, nous avons identifié une augmentation de signes de stress cellulaires tels que la présence de boursoufflures dans l'axone présynaptique ainsi qu'une fragmentation de la JNM chez les souris miR-C9orf72 âgées (15 et 23 mois).

Nous avons évalué si la perte de fonction du gène pouvait causer une neurodégénérescence ou une gliose. Le nombre de neurones est conservé au niveau du cortex frontal et du cortex moteur et nous n'avons pas identifié d'activation microgliale ou d'astrogliose dans ces mêmes régions.

Ensuite, nous nous sommes intéressés à l'état des protéines TDP-43 et p62 dans le cerveau de souris miR-C9orf72. En effet, dans le cerveau et la moelle épinière de patients FTD et ALS, nous observons une relocalisation cytoplasmique de la protéine TDP-43 phosphorylée ainsi que son agrégation alors que chez les individus sains, cette protéine a une localisation nucléaire. De même, des agrégats contenant la protéine p62 sont retrouvés dans le cerveau de patients DFT portant la mutation *C9ORF72*. Les souris miR-C9orf72 ne présentent pas d'agrégation cytoplasmique de protéine TDP43. Cependant, nous avons observé une augmentation du nombre de cellules corticales contenant une accumulation de p62 chez ces souris. Nous avons identifié que ces structures étaient aussi positives pour le marqueur de lysosomes Lamp1 dans les neurones et les microglies.

En conclusion, l'étude des souris miR-C9orf72, présentant un knockdown du gène *C9orf72* a montré que la perte de fonction est impliquée dans l'apparition de symptômes propres à la DFT. Au niveau cellulaire, cette perte de fonction conduit à une altération de la voie autophagique dont le rôle délétère dans la DFT reste à déterminer. De plus, nous avons montré que la perte de fonction de *C9orf72* n'est pas un élément déterminant pour causer la mort neuronale ou induire une agrégation de la protéine TDP-43, suggérant que les

mécanismes de toxicité de l'ARN et des agrégats de dipeptides pourraient jouer un rôle majeur dans ces aspects.

D'après les différents travaux publiés à ce jour, il apparaît que des souris hétérozygotes ou avec un knock-out neuro-spécifique pour *C9ORF72* ne développent pas d'atteinte motrice ou de diminution de la survie. De plus, des souris knock-out ubiquitaires pour *C9orf72* présenteront une altération spécifique du comportement social à partir de l'âge de 6 mois. Contrairement aux souris miR-*C9orf72* et aux souris hétérozygotes, les souris knock-out pour le gène *C9orf72* présentent aussi une immunité périphérique anormale à partir de l'âge de 2 mois, avec le développement d'une splénomégalie et d'une lymphoadénopathie. Chez ces souris, les macrophages et les microglies présentent un défaut au niveau du trafic lysosomal tardif. Ces observations relient la fonction de la protéine C9ORF72 à la voie autophagique en tant qu'échangeur GDP/GTP, et une déficience dans son expression sera à l'origine d'une défaillance au niveau de la maturation lysosomes-endosomes, conduisant à une atteinte immunitaire en périphérie et dans le système nerveux central. Néanmoins, des études supplémentaires sont encore nécessaires pour identifier la fonction de la protéine C9ORF72 et les effets spécifiques à sa déficience partielle chez les patients.

L'étude de souris modélisant le gain-de-fonction toxique des ARN mutants et des dipeptides répétés a montré que l'expansion GGGGCC peut causer l'altération de comportements moteur et cognitif avec une perte neuronale et une délocalisation de la protéine TDP-43 lorsque celle-ci est surexprimée. Les mêmes observations n'ont pas été reproduites quand l'expansion est exprimée à un niveau physiologique, même si des foci d'ARN et des agrégats de dipeptides sont présents, suggérant qu'un facteur additionnel est requis pour induire la neurodégénérescence. De ce fait, nous émettons l'hypothèse que la perte de fonction de *C9ORF72* est un élément requis pour mener au déséquilibre cellulaire causant une sensibilité accrue aux toxicités de l'ARN et des dipeptides produits par l'expansion GGGGCC. Les souris miR-*C9orf72*, présentant une déficience en *C9orf72* semblable à celle des patients, pourraient ainsi être utilisées comme modèles pour tester les effets toxiques spécifiques de l'ARN mutant et des dipeptides répétés. Ainsi, ce nouveau modèle pourra donc servir comme outil pour déchiffrer les mécanismes moléculaires de la pathologie et identifier des cibles thérapeutiques spécifiques aux c9FTD/ALS.

# Index

<b>FIGURES INDEX</b>	<b>X</b>
<b>TABLE INDEX</b>	<b>XI</b>
<b>ABBREVIATIONS</b>	<b>XII</b>
<b>INTRODUCTION</b>	<b>14</b>
<b>I. GENERAL INTRODUCTION</b>	<b>14</b>
<b>II. NEURODEGENERATIVE DISEASES</b>	<b>16</b>
1. PROTEIN AGGREGATION IN NEUROLOGICAL DISEASE	16
1.1. Inclusion bodies and aggresomes	19
1.1.1. Kinetics of aggregate formation	19
1.1.2. Inclusion bodies	21
1.2. Awry autophagy	23
1.3. p62, a link between autophagy and the ubiquitin proteasome system	26
1.4. Ubiquitin proteasome system and the unfolded protein response	27
1.5. Nucleocytoplasmic shuttling dysfunction	30
2. RNA METABOLISM ALTERATION IN NEUROLOGICAL DISEASE	32
2.1. Molecular mechanisms leading to repeat expansion formation	34
2.2. Molecular mechanisms leading to repeat-mediated toxicity in the case of non-coding expansions	35
<b>III. FRONTOTEMPORAL DEGENERATION AND AMYOTROPHIC LATERAL SCLEROSIS: TWO ENDS OF A SAME DISEASE SPECTRUM</b>	<b>39</b>
1. FEATURES OF FRONTOTEMPORAL DEMENTIA	39
1.1. Epidemiology and clinical features	39
1.2. Diagnosis	41
1.3. Neuropathology	42
1.4. Genetics	45
2. FEATURES OF AMYOTROPHIC LATERAL SCLEROSIS	48
2.1. Epidemiology, clinical features and genetics	48
2.2. Diagnosis	50
2.3. Neuropathology	52
3. HOW HAVE A DEMENTIA AND A MOTOR NEURON DISEASE BEEN LINKED TOGETHER?	55
4. THE <i>C9ORF72</i> REPEAT EXPANSION, TYING UP FTD AND ALS	56
4.1. Transcripts and protein variation in <i>C9orf72</i> carriers	57
4.2. Inclusions in <i>C9orf72</i> patients	58
<b>IV. UNDERLYING PHYSIOPATHOLOGICAL MECHANISMS IN FTD AND ALS</b>	<b>60</b>
1. IMPLICATIONS OF TDP43 IN FTD AND ALS AND ITS ROLE AS AN MRNA BINDING PROTEIN	60
1.1. <i>TARDBP</i> gene and TDP43 protein	61
1.2. Pathological TDP43	65
1.2.1. <i>TARDBP</i> mutations	65
1.2.2. TDP43 proteinopathies and aggregation	66
1.2.3. TDP43 hyperphosphorylation	67
1.2.4. TDP43 ubiquitylation	68

1.2.5. TDP43 mislocalization	68
1.2.6. TDP43 pathological models	69
1.2.7. TDP43 mediated neurodegeneration	71
2. C9ORF72 IN ALS AND FTD PHYSIOPATHOLOGY	75
2.1. C9orf72 gene and protein	75
2.2. Pathological C9orf72	77
2.2.1. Repeat-expansion toxic gain-of-function contribution to disease pathogenesis	77
2.2.2. C9orf72 loss-of-function contributions to disease pathogenesis	84
2.3. Implications of the C9orf72 repeat expansion in cellular pathways: what do we know so far?	87
2.3.1. C9orf72 repeat expansion causes nucleolar stress	89
2.3.2. C9orf72 repeat expansion causes splicing defects	89
2.3.3. C9orf72 repeat expansion causes nucleocytoplasmic transport impairment	90
<b>V. AIMS OF THE STUDY AND CHOICE OF MODEL</b>	<b>94</b>

**RESULTS: PARTIAL C9ORF72 LOSS OF FUNCTION IN MICE LEADS TO MILD BEHAVIOR ANOMALIES  
EVOKING FTD-LIKE SYMPTOMS WITHOUT MOTOR NEURON DISEASE (LOPEZ-HERDOIZA ET. AL)** 96

**DISCUSSION AND FUTURE PERSPECTIVES** 140

<b>I. ROLE OF THE C9ORF72 LOSS-OF-FUNCTION MECHANISM IN THE DISEASE COURSE: GAINING INSIGHT FROM THE MIR-C9ORF72 MOUSE MODEL</b>	<b>140</b>
<b>II. BEHAVIOR EVALUATION OF MICE MODELS AND CORRELATIONS TO CLINICAL PATHOLOGY</b>	<b>146</b>
<b>III. HYPOTHESIS OF UNDERLYING MECHANISMS DRIVING NEURODEGENERATION IN C9FTD/ALS</b>	<b>150</b>
1. PROTEIN HOMEOSTASIS, AGGREGATION AND TDP43 PATHOLOGY	150
2. MICROGLIA C9ORF72 IMPLICATIONS	151
3. ALS/FTD MECHANISMS NOT DIRECTLY RELATED TO THE C9ORF72 LOSS-OF-FUNCTION... OR ARE THEY?	153
3.1. Nucleocytoplasmic transport	153
3.2. RNA metabolism	154
3.3. Prion-like spreading and TDP43	155

**CONCLUSION** 156

**REFERENCES** 158

**ANNEXES** 186

**ABSTRACT**

## Figures index

<b>Figure 1</b> Formation of structured protein aggregates.	20
<b>Figure 2</b> Model for aggresomes formation.	21
<b>Figure 3</b> Autophagy pathway	25
<b>Figure 4</b> ER stress-mediated apoptosis and the Unfolded Protein Response (UPR)	29
<b>Figure 5</b> Schematic representation of nucleocytoplasmic shuttling	31
<b>Figure 6</b> Coding and non-coding expansions associated to neurodegenerative diseases	34
<b>Figure 7</b> Roles of RBPs in RNA metabolism	36
<b>Figure 8</b> RNA interference and the RISC complex	37
<b>Figure 9</b> Clinical symptoms of FTD	40
<b>Figure 10</b> Overview of FTD alterations – Brain images	42
<b>Figure 11</b> Pathological inclusions of TDP43 found in FTD and ALS	43
<b>Figure 12</b> Schematic representation of TDP43, p62 and DPR inclusions type	44
<b>Figure 13</b> Possible diseases mechanisms of the C9orf72 repeat expansion.	48
<b>Figure 14</b> ALS common symptoms	51
<b>Figure 15</b> Tools for ALS differential diagnosis	52
<b>Figure 16</b> The “dying-forward” and “dying-back” motor neuron hypothesis.	54
<b>Figure 17</b> Schematic representation of the FTD and ALS continuum	55
<b>Figure 18</b> Physiological roles of TDP43 (and FUS)	63
<b>Figure 19</b> TDP43 gene representation and localization of known mutations	65
<b>Figure 20</b> TDP43 mutations and their functional effects	66
<b>Figure 21</b> Stages of TDP43 spreading and deposition in FTD (left) and ALS (right)	72
<b>Figure 22</b> TDP43 toxicity models	74
<b>Figure 23</b> C9orf72 gene and protein isoforms	76
<b>Figure 24</b> RBPs and the C9orf72 repeat expansion	78
<b>Figure 25</b> RAN translation products of the C9orf72 repeat expansion	80
<b>Figure 26</b> RAN products associated toxicity	81
<b>Figure 27</b> Models of nucleocytoplasmic transport impairment mediated by GGGGCC toxicity	92
<b>Figure 28</b> Behavioral characterization of miR-C9orf72 mice	95
<b>Figure 29</b> Behavior alterations in the absence of C9orf72	142
<b>Figure 30</b> Mouse models of FTD and associated behavioral assessments	147
<b>Figure 31</b> Saliency network in human and mice	148

## Table index

<b>Table 1</b> <i>Aggregating proteins in neurodegenerative diseases</i>	18
<b>Table 2</b> <i>Identified genes causing familial FTD</i>	46
<b>Table 3</b> <i>Identified genes causing familial ALS</i>	50
<b>Table 4</b> <i>Pathological mechanism of the C9orf72 expansion Adapted from Haeusler et al. (2016)</i>	88
<b>Table 5</b> <i>Behavior assessment of mouse models</i>	146

## Abbreviations

3-MA	3-methyladenine
AD	Alzheimer disease
<b>ALS</b>	Amyotrophic Lateral Sclerosis
ALS-PDC	Amyotrophic Lateral Sclerosis-Parkinsonisms-Dementia Complex
ANG	Angiogenine
APOA3	Apolipoprotein A3
ASOs	Antisense oligonucleotids
ATF6	Autophagy factor 6
ATXN2	Ataxin 2
<b>BAC</b>	Bacterial artificial chromosome
BMAA	β-methylamino-L-alanin
BMI	Body mass index
bvFTD	Behavioral variant frontotemporal dementia
<b>C9-L</b>	C9orf72 long isoform
C9ORF72	Chromosome 9 open reading frame 72
<b>C9-S</b>	C9orf72 short isoform
CELF	CUG-BP and ETR-3-like factors
CFTR	Cystic Fibrosis Transmembrane conductance Regulator
CHMP2B	Charged mutivesicular body protein 2B
CMT2B	Charcot Marie Tooth type 2B
CTFs	C terminal fragments
DENN	Differentially expressed in normal and neoplastic cells
DM1 - 2	Myotonic dystrophy 1 -2
DN	Dystrophic neurites
<b>DPRs</b>	Dipeptide repeats
EMG	Electromyogramme
ER	Endoplasmic reticulum
ERAD	Endoplasmic-reticulum-associated protein degradation
ESCRT	Endosomal sorting complex required for transport
<b>FTD</b>	Frontotemporal dementia
<b>FUS</b>	Fused in Sarcoma
FXTAS	Fragile X-associated tremor/ataxia syndrome
GEF	Guanine nucleotide exchange <i>factor</i>
HD	Huntington disease
hNFL	Human neurofilament
hnRNP	Heterogeneous nuclear ribonucleoproteins
<b>HRE</b>	Hexanucleotide repeat expansion
hsp	heat shock protein
IPSC	Induced pluripotent stem cell
LIR	LC3-interacting region
MAPT	Microtubule-associated protein tau
MBLN	Muscleblind
<b>miRNA</b>	microRNA
MND	Motor neuron disease

MRI	Magnetic resonance imaging
mTOR	Mammalian target of rapamycin
NCI	Neuronal cytoplasmic inclusion
NCT	Nucleocytoplasmic transport
NES	Nuclear export signal
nfvPPA	Non fluent variant primary progressive aphasia
NIIS	Neuronal intranuclear inclusions
NLS	Nuclear localization signal
NPC	Nuclear pore complex
Nups	Nucleoporins
OPTN	Optineurin
PD	Parkinson disease
PET	Positron emission tomography
PGRN	Progranulin
PrPSC	Prion proteins
Rab-GEFs	Rab guanosine exchange factors
<b>RAN</b>	Repeat associated non ATG translation
<b>RBPs</b>	RNA binding proteins
RISC	RNA-induced silencing complex
RNAi	RNA interference
RNP	RNA molecules linked to ribonucleoproteins
RRM	RNA recognition motifs
SCA	Spinocerebellar ataxia
SMN	Survival of motorneuron
SOD1	Super oxide dismutase 1
SQSTM1	Sequestosome 1
STAMBP	Signal-transducing adaptor molecule binding protein
svPPA	semantic variant primary progressive aphasia
TDP43	TAR DNA-binding protein 43
TNF	Tumor necrosis factor
TRAF6	TNF associated factor 6
UBA	Ubiquitin associated
UBQL2	Ubiquilin-2
UPR	Unfolded protein response
UPS	Ubiquitin proteasome system
UTR	Untranslated region
VEN	Von Economo neurons
XPO5	Exportin 5



## Introduction

### I. General introduction

Neurodegeneration is the generic term used to describe progressive loss-of-function or death of neurons. Neurodegenerative diseases occur as a result of progressive degeneration and/or cell death throughout different, non-fully characterized mechanisms. As world population lifespan increases, so does the prevalence of neurodegenerative diseases such as Alzheimer's Disease (AD), Parkinson's Disease (PD), Huntington's Disease (HD), Amyotrophic Lateral Sclerosis (ALS), Fronto-Temporal Dementia (FTD), among others.

Causes for these pathologies are mostly unknown. Treatments proposed nowadays tend only to alleviate symptomatology, and not to treat the essential deleterious processes. The increased identification of loci and genes implicated in familial forms has provided answers on the underlying pathological mechanisms allowing for the development of new treatments targeting specific impaired pathways. The need to develop more accurate models to study identified pathways at a cellular and molecular level has never been more important, as they will also be key to test for therapies and to identify new, more efficient therapeutic targets.

As research advances, so does our knowledge on mechanisms leading to neurodegeneration, and surprisingly, similarities relating one disease to another on a sub-cellular level are often found. Most of these diseases present pathological hallmarks such as protein aggregation due to misfolding or atypical protein assembly, as is the case in AD with  $\beta$ -amyloid plaques accumulation,  $\alpha$ -synuclein aggregates in PD, or TAR-DNA binding Protein 43 (TDP43) aggregates in FTD and ALS. Another common mechanism corresponds to alterations in RNA metabolism. RNA toxicity was firstly described in myotonic dystrophy 1 and myotonic dystrophy 2 (DM1 and DM2), a multisystemic disorder caused by the abnormal expansion of a non-coding trinucleotide sequence (Ranum and Day, 2002). In several cases, both protein aggregation and RNA toxicity seem to occur in parallel as is the case with hexanucleotide GGGGCC repeat expansion (HRE) in *C9ORF72* a mutation responsible for both ALS and FTD (C9FTD/ALS). Little is known about the pathomechanisms of the HRE in C9FTD/ALS, and several hypotheses implicating production of a toxic RNA, peptide-aggregates toxicity or loss-of-protein function have been proposed. The C9ORF72 HRE is the most common cause of both ALS and FTD, and of the syndrome ALS/FTD. These two apparently different neurodegenerative diseases are related by numerous similarities including clinical presentation, mutated genes and identical neuropathological lesions with the presence of TDP43 inclusions. The course through which

the mutation of *C9ORF72* drives pathogenicity remains elusive. However, the development of multiple and complementary animal models reproducing punctual aspects of the mutation has contributed to clarification of the underlying mechanisms leading to disease development or evolution. The study of familial history, clinical data and neuropathology of patients has allowed the establishment of relationships between different mechanisms, but also has given a broader picture of neurodegenerative diseases showing that in some cases, one syndrome may overlap with another.

In this work, I will present my research in mechanisms involved in neurodegeneration based on the study of the *C9ORF72* mutation. As previously mentioned, several mechanisms seem to co-exist in patients carrying the HRE, which make it an interesting model for studying pathways common to neurodegenerative diseases. To present this work, I will initially introduce background of mutual underlying mechanism in neurodegenerative diseases focusing on protein aggregation and RNA metabolism alterations and how their influence may lead to neurodegeneration (Chapter I). I will then follow to a global presentation of FTD and ALS, two related but clinically different syndromes. I will pay a particular attention to the implication of the *C9ORF72* mutation to both of these pathologies (Chapter II). Thirdly, I will merge the concepts presented in the two initial chapters to review molecular mechanisms and how they may lead to FTD and/or ALS. For this, I will analyze knowledge on TDP43, the main aggregating protein in FTD and ALS, which is also an RNA binding protein (RBP) directly implicated in RNA metabolism and then review the different mechanisms driven by the *C9ORF72* mutation (Chapter III). Finally, I will present the aims of my PhD project for which on the *C9orf72* loss-of-function mechanism and its implications in ALS/FTD (Chapter IV).

## **II. Neurodegenerative diseases**

### **1. Protein aggregation in neurological disease**

Maintaining homeostasis is one of the major processes the cell has to carry out. The need for cellular adaptation emerges when fluctuations in the environment or of its intrinsic state occur. This will lead the cell to re-organize processes and functions accordingly. Mechanisms regulating cellular survival in aged or degenerative cells remain still poorly understood, but knowledge points toward aged-related differences between mechanisms. The neurodegenerative cell will yet have supplementary alterations to adapt adding to aging and exterior fluctuations, leading to a possible overflow in adaptive mechanisms. From this, the analysis of the brain of patients with different neurodegenerative disorders showed the presence of intracellular accumulation of protein aggregates, suggesting the cell is not able to compensate for variations. Presence of protein aggregates is a common feature of these diseases and their toxicity has been hypothesized as they may eventually lead to degeneration and death of cells (Miller and Kaplan, 2011). On the contrary, in normal conditions a rapid controlled apoptotic suicide is commonly observed when the cell is confronted to deprivation or stressful conditions (Nikoletopoulou et al., 2013). Increasing evidence indicates that, in the aged or degenerative nervous system, prolonged exposure to relatively minor insults might lead to cumulative neuronal oxidative stress and/or DNA damage, which in turn causes neurons to atrophy and degenerate.

Protein folding is essential for cellular protein homeostasis, allowing biological pathways to ensure cellular viability. Correctly folded proteins acquire a native three-dimensional functional structure that has to be maintained throughout the lifetime of each protein. Membrane and secretory proteins folding occurs mainly in the Endoplasmic Reticulum (ER) lumen, which allows for post-translational modifications due to its oxidizing conditions and numerous resident chaperons (Parakh and Atkin, 2016). Cytoplasmic proteins do not normally transit the ER and thus, rarely present disulphide bond formation, which are important for the structural stability of proteins and endorse for multi-protein complexes formation. Several E3 ligases ensure protein folding before the protein has left the ribosome, and chaperones from the hsp family will ensure correct protein trafficking and avoid aggregation (Amm et al., 2014). Protein misfolding is common to all proteins; mutations, heat, oxygen radicals, heavy metal ions and other stresses can enhance abnormal conformations and disturb proper folding and even alter already properly folded proteins. At

least, 30% of newly synthesized proteins are misfolded (Amm et al., 2014; Parakh and Atkin, 2016). To ensure proper protein folding, under normal conditions, several cellular protein quality control systems monitor newly synthesized proteins that maintain protein homeostasis, also known as proteostasis (Amm et al., 2014). When this system fails to correctly refold proteins, they will be directed to degradation by the proteasome, particularly the Ubiquitin Proteasome System (UPS) or autophagy system.

During protein misfolding, conformational changes occur in the hydrophobic core of the protein, leaving it exposed and prone to aggregation. “Conformational diseases” are a group of disorders characterized by the conversion of the tertiary structure of a protein to a different, newly stable form, resulting in cellular perturbation. If the misfolded protein is not shuttled towards degradation, it can oligomerize and aggregate. These latter processes could cause toxic effects by (1) a loss-of-function effect due to the lack of biological activity from the misfolded protein or (2) a toxic gain of function of the protein in its new form, both of these effects eventually leading to an altered cellular survival followed by cellular dysfunction and death in the long term. Intracellular and extracellular protein aggregation both are linked to several diseases and are commonly observed in neurodegenerative diseases (**Table 1**) (Hardy and Orr, 2006; Kopito, 2000; Ogen-Shtern et al., 2016; Parakh and Atkin, 2016).

**Table 1** Aggregating proteins in neurodegenerative diseases

Disease	Protein	Characteristic pathology	Pathologic species
<b>Parkinson's disease (PD)</b>	Alpha synuclein	Intracellular Membrane associated	Tetramers
<b>Alzheimer's disease (AD)</b>	Tau	Intracellular	Fibrils
	Beta-sheets	Extracellular	Dimers Oligomers
<b>Huntington's disease (HD)</b>	Huntingtin (Htt)	Intracellular Intranuclear	Oligomer Aggregates
<b>Amyotrophic lateral sclerosis (ALS)</b>	TDP43	Intracellular	Aggregates
	FUS		
	SOD1	Intracellular	Dimers Aggregates
<b>Frontotemporal dementia (FTD)</b>	TDP43	Intracellular	Aggregates
	FUS	Intracellular	Aggregates
	Tau	Intracellular	Tangles

*Adapted from Christopher Ross & Michelle Poirier (2004)*

Accumulating proteins are pathological features of each disorder and proteins that misfold in each case are specific to a respective condition and a lesion site, although similarities in protein aggregation can occur in multiple diseases. Such is the case of TDP43 pathology in both ALS and FTD, or Tau aggregation in AD and FTD (Eftekharzadeh et al., 2016; Ogen-Shtern et al., 2016). Even though clinical manifestations are different, these “aggregating protein assemblies” eventually leading to neuronal dysfunction may share common pathways in neurodegenerative diseases. Yet, our knowledge of underlying mechanisms is still limited and it has become essential for us to increase our understanding of initial events leading to disease. From a molecular level, we need to characterize the triggers and processes of protein misfolding, aggregation and formation and the potential underlying cell toxicity mechanisms. Also, the transition between monomeric and aggregated – passing through partially unfolded – states has yet to be elucidated. From a physiological level it is important to understand whether these aggregates affect cell homeostasis and survival by altering cytoplasmic function due to abnormal interactions of misfolded or mutated proteins with common partners leading to toxicity, or transcriptional deregulations due to the

aggregates themselves or to RNA modification, or altering nucleocytoplasmic shuttling, or causing nuclear function deregulation with the culmination in degeneration.

## **1.1. Inclusion bodies and aggresomes**

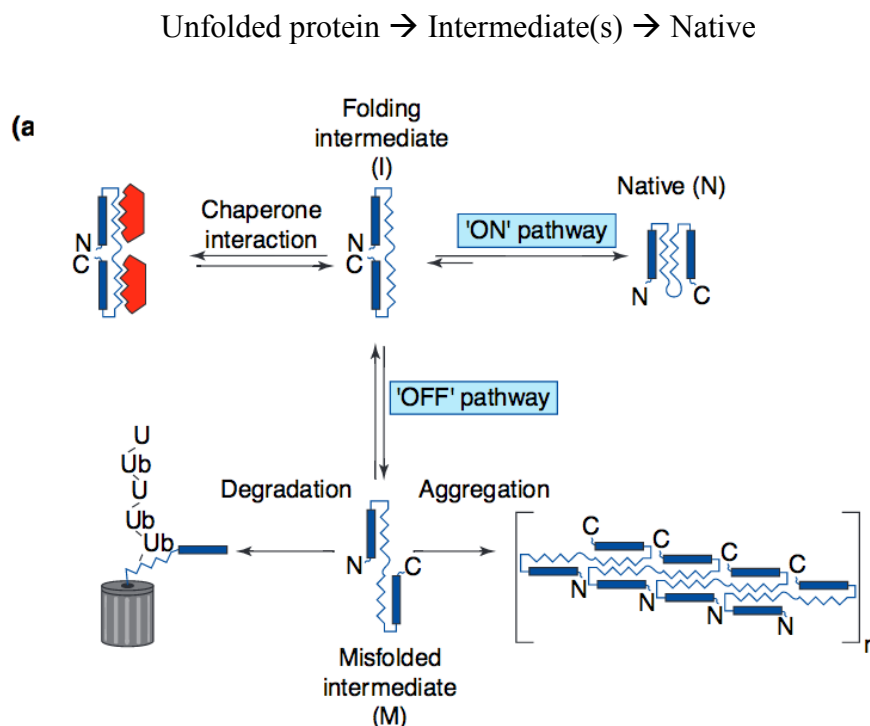
### **1.1.1. Kinetics of aggregate formation**

Protein aggregates are oligomeric complexes of non-native conformers that assemble from altered interactions among structured, kinetically trapped intermediates of the protein-folding pathway. Protein aggregation is caused by alterations in the protein primary structure produced by mutations, RNA modification, translation mis-incorporation or by partial unfolding during thermal or oxidative stress (Kopito, 2000). These factors will cause hydrophobic domains to be exposed allowing them to interact with other proteins and sequester them in an autocatalytic process, where misfolded oligomers can act as “seeds” to form larger misfolded structures (Parakh and Atkin, 2016). Structured protein aggregates are called amyloid assemblies and tend to be insoluble and metabolically stable under physiological conditions. Unstructured protein aggregates are called amorphous (Kopito, 2000). The suggested pathway leading to structural transition indicates that the natively folded protein monomers becomes partially unfolded and transit into an unstable disordered state from where it adopts a misfolded state which will enable assembly into energetically stable complexes (Eftekharzadeh et al., 2016).

Initially, it was believed that the native state of proteins was specified only by the amino acid sequence, and that folding was neither templated nor assisted by other factors. But, in 1972, it was shown that proteins have at least two states: a native state and an amyloid state, which is more stable (Anfinsen, 1972; Pauling et al., 1951). Amyloids are formed by nucleation, a templating process with strong thermodynamic effects that allows an energetic funnel to drive protein assembly (Bryngelson et al., 1995; Govindarajan et al., 2006). Once the nucleus or “seed” is formed, there is an exponential growth of aggregation or seeded polymerization, as proteins tend to stay in the lowest free energy state (Walker et al., 2013). Properly folded proteins have their hydrophobic regions buried within the structure, ensuring the lowest possible energy state, but misfolded proteins have exposed hydrophobic regions (Ogen-Shtern et al., 2016). Chaperons of the “quality control” (QC) machinery ensure that protein aggregates do not accumulate in unstressed cells by selectively degrading inappropriately folded proteins before they can aggregate. These molecular chaperons interact transitorily with protein intermediates concealing hydrophobic surfaces from forming intra- or

intermolecular interaction and block or and minimize aggregation (Kopito, 2000; Ogen-Shtern et al., 2016). By this, they ensure correct protein folding and the maintenance of protein homeostasis (known as proteostasis), which are fundamental for cell viability (**Figure 1**).

As a last resort, cellular pathways will cause the assembling of beta sheet-containing domains into amyloid fibrils in which the hydrophobic surfaces will be less exposed to damaging interactions (Ogen-Shtern et al., 2016). Accumulation of misfolded proteins can arise when these quality control pathways malfunction. Even if the formation of amyloid structures correlates with cell death, the relation between aggregates and neuronal loss has not been fully elucidated. Nevertheless, the question remains to whether aggregates are directly pathogenic or if it is the transition from natively folded proteins into amyloid that reflects or triggers toxicity.



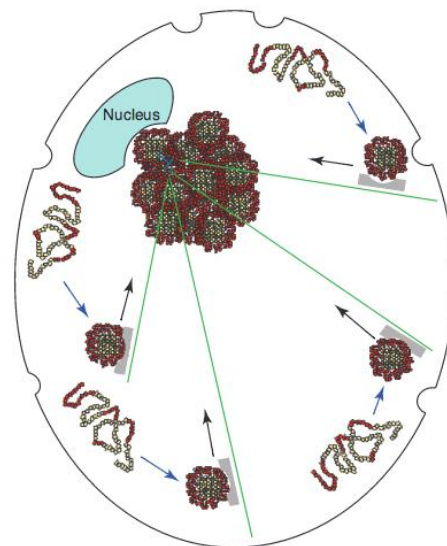
**Figure 1** Formation of structured protein aggregates.

The overall efficiency of protein folding is the proportion of intermediates that assume a native conformation. Intermediates can assume misfolded, off-pathway conformation, which are prone to oligomerize into non-native aggregates. The ubiquitin-proteasome pathway degrades protein intermediates before they can aggregate. *Adapted from Kopito (2000)*

### 1.1.2. Inclusion bodies

Inclusion bodies are intracellular foci, and in the case of neurodegenerative diseases, aggregated proteins mainly form them. Besides the major aggregated protein species, immunohistochemical analysis have revealed the presence of molecular chaperones, including heat shock proteins (hsp Hsc70, Hsp40), the proteasome subunits 19S and 26S, ubiquitin, intermediate filament proteins (Kopito, 2000; Ogen-Shtern et al., 2016). Whether the composition of these inclusion bodies reflects malfunction of the protein degradation and folding machinery or variable activities of cellular deubiquitinating enzymes has not yet been elucidated. Inclusion bodies are present in low copy number, usually one per cell and, in the case of neurodegenerative diseases they are accompanied by displaced and abnormally phosphorylated intermediate filaments (neurofilaments).

The mechanisms leading to formation of inclusion bodies in degenerative neurons represent an intense field of study. A common feature of trapped proteins is the presence of intrinsically disordered domains that could facilitate non-covalent hydrophobic bonding or ionic interactions. It is now greatly supported that the formation of inclusion bodies in animal cells can take place by fusion of individual protein aggregates into a limited number of foci



**Figure 2** Model for aggresomes formation.

Small aggregates formed at the cell periphery are derived along microtubules tracks (green lines) on retrograde motors (grey) to a juxtannuclear, pericentriolar localization. *Adapted from Kopito (2000)*



driven by active retrograde transport of the misfolded proteins on microtubules (**Figure 2**), contrary to the idea that they were formed by a diffusion process (García-Mata et al., 1999; Johnston et al., 1998; Kopito, 2000).

Inclusion bodies that are dependent on active microtubule transport are called “aggresomes”. Forms of misfolded or misassembled proteins that are prone to form inclusion bodies such as presenilin 1 (Johnston et al., 1998), peripheral myelin protein PMP22 (Notterpek et al., 1999) or ALS causing mutation in superoxide dismutase 1 (SOD1) enzyme (Johnston et al., 2000) are located at the pericentriolar region, suggesting a central role for this region in aggresomes formation. This region is known to contain proteins responsible for microtubule nucleation and anchoring. Like so, it is not surprising to see that protein aggregates accumulation in the pericentriolar region will result in expansion of the centrosome, disruption of Golgi’s normal architecture and disorganization of microtubules (Quintyne et al., 1999). The Golgi apparatus function depends on dynamic vesicular traffic (Lippincott-Schwartz, 1998), and Golgi cisternae displacement is a common feature in neurons of AD brains and spinal cords from ALS patients and mouse models of ALS (Gonatas et al., 1998), relating microtubule-based transport of aggregating proteins to cellular mechanisms that could be implicated in neurodegeneration (Kopito, 2000).

On the contrary, a protector effect of inclusion bodies has been also suggested, as they may have a role in protein homeostasis and act as cellular bins reducing levels of toxic intermediate forms, as the formation and accumulation of protein aggregates also takes place during normal aging (Cuanalo-Contreras et al., 2013). Protein turnover is essential for maintaining cellular homeostasis by removing defective proteins, by contributing to the pool of amino acids required for protein synthesis within limited nutriment availability and for allowing an optimal equilibrium of proteins themselves. Proteostasis, or protein homeostasis is responsible for the synthesis, folding and turnover of proteins (Balch et al., 2008), and pathways including the unfolded protein response (UPR), the UPS, autophagy and encapsulation of damaged proteins in aggresomes maintain the balance in the cell. Studies and genetic evidence showed that 30% of all newly synthesized cellular proteins are defective ribosomal products that will be degraded shortly after their synthesis and that normal cells must cope with a continuous flux of misfolded proteins that must be degraded (Nedelsky et al., 2008; Zhou and Schekman, 1999). For this, the proteasome active sites need to be able to directly interact with protein aggregates in order to degrade them. Chaperons will help the proteasome system by unfolding aggregates, but the system can be overwhelmed as the imbalance tilts towards aggregation. Whether protein aggregation will be able to over-drown

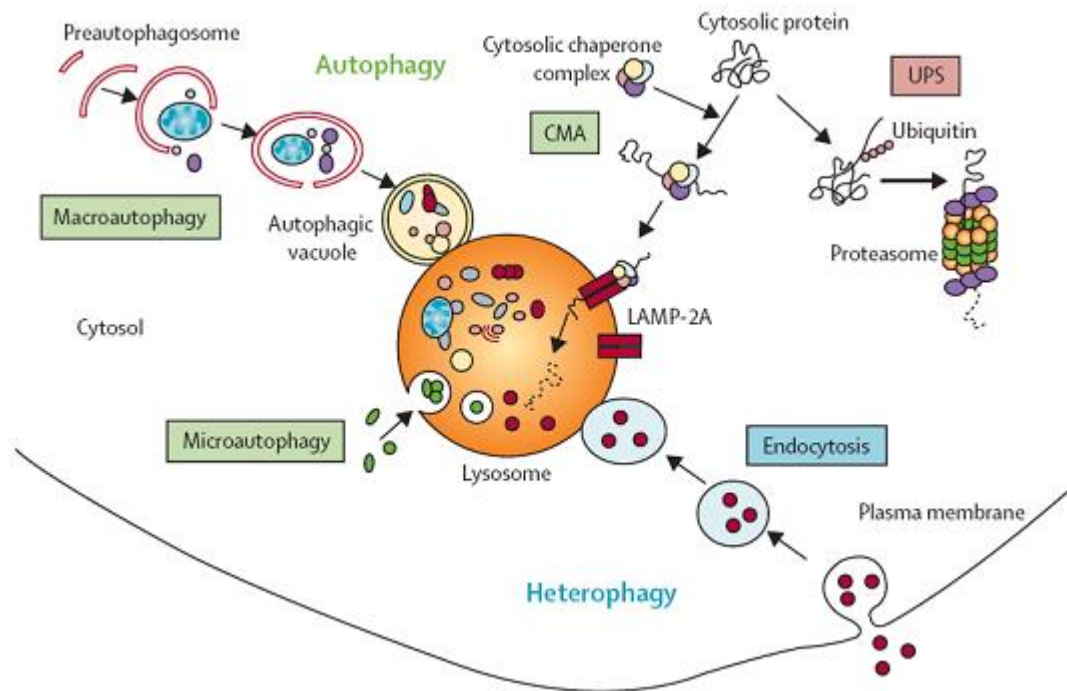
the cell will depend upon the competition between the effective degradation of misfolded substrates and the aggregation process itself (Kopito, 2000). With aging, degrading mechanisms do not perform equally and as so, protein aggregates are able to better stabilize themselves, thus tending to accumulate (Taylor and Dillin, 2011).

## 1.2. Awry autophagy

One of the main mechanisms of protein degradation is through autophagy. The autophagic route facilitate their disposal of aggregates by delivering them to a centralized region, as can be the pericentriolar region for example (Johnston et al., 1998). Evidence suggest that autophagy serves two major purposes in mammalian cells: (1) to provide an amino-acid supply under poor environmental conditions, which is known as “adaptive autophagy” and (2) to continually degrade proteins in the cytoplasm, which is known as “constitutive autophagy”. This process is the major process through which long-lived cytoplasmic components, such as excess or damaged organelles and aggregate-prone proteins, will be degraded by lysosomes (Komatsu et al., 2007). Three main types of autophagy have been characterized so far: (1) chaperon-mediated autophagy, which involves specific protein recognition and delivery to lysosomes, (2) microautophagy, which is poorly understood and (3) macroautophagy (referred here as autophagy) (**Figure 3**). Knowledge of chaperone-mediated autophagy and microautophagy implications in neurodegeneration remains poorly characterize, so I will not explore them in this work. On the other hand, autophagy implications in neurons have been studied deeper. Neurons are highly metabolically active post-mitotic cells. As they cannot dilute toxic waste burdens by cell division, they may try to get rid of aggregating proteins throughout autophagy. Constitutive autophagy activity in the brain is low when compared to other tissues, but it plays a crucial role as altering its function causes major neurodegenerative phenotypes (Mizushima, 2004). Mechanistically, large cytoplasmic regions, organelles and peripherally formed aggregates are engulfed within a double membrane vesicle, probably by fusion with an endosome, to create an autophagosome. In turn, the autophagosome will then fusion with lysosomes leading to turnover and recycling of cellular constituents (Komatsu et al., 2006; Wong and Cuervo, 2010). Formation of the autophagosome is regulated by the beclin VPS34 and the mTOR kinases complexes (Taylor and Dillin, 2011). When the pathway becomes overwhelmed, the continual delivery of protein aggregates might turn the deliver site into a cellular junkyard, as is the case in affected brains from patients of AD, PD, ALS, FTD and polyQ diseases in which neurons present an

abnormal amount of autophagosomes (Nedelsky et al., 2008; Wong and Cuervo, 2010). Accordingly, impairment of autophagy causes neurodegeneration in animal models. Autophagy deficient mice develop progressive deficits in motor function including an ataxic walking pattern, limb clasp reflexes and tremor. Modification of autophagy pathways also alters phenotype in animal models of neurodegeneration, either by reducing or accelerating degeneration (Hara et al., 2006; Komatsu et al., 2007, 2006; Nedelsky et al., 2008). Implications of autophagy have been further accepted when mutations or defects in autophagy-lysosomal function have been shown to have an impact on familial neurological diseases. For example mutation in a GTPase exchange factor that participates in trafficking autophagosomes and fusion with lysosomes called Rab7, result in dominantly inherited axonal neuropathy Charcot-Marie-Tooth type 2b (CMT2B) (Nedelsky et al., 2008) and autophagy related genes *OPTN*, *SQSTM1* and *CHMP2B* will lead to ALS or FTD (Fecto et al., 2011; Le Ber et al., 2013; Maruyama et al., 2010; Skibinski et al., 2005).

Previous examples concern mutations in the autophagy pathway leading to neurodegeneration. A separate question implicates the role of autophagy in neurodegenerative diseases caused by mutations in genes unrelated to the autophagy function itself. Upregulation of autophagy can be induced by pharmacologically inhibiting the kinase mTOR (mammalian target of rapamycin). The mTOR pathway regulates a variety of cellular functions including cellular proliferation, motility, and survival, among others. Failure in this pathway has been associated with several pathologies including autism and AD (Rosner et al., 2008; Tang et al., 2014). Thus, mutations that affect the mTOR pathway can alter autophagy and eventually, lead to neurodegeneration (Damme et al., 2014). Interestingly, the other autophagy pathway called microautophagy, which comprises the direct invagination of lysosomal membrane with cytoplasmic components, is also under the control of the mTOR pathway (Bitto et al., 2014), suggesting an interlace of the different types of autophagy pathways. Inducing macro autophagy by mTOR pharmacological inhibition has been shown to accelerate clearance of protein aggregates in vitro and protect against neurodegeneration in animal models, particularly delaying ALS progression in mouse models (Nedelsky et al., 2008; Rea et al., 2014; Wong and Cuervo, 2010). On the same line, TDP43 aggregation, the aggregating protein in both FTD and ALS, can be induced by inhibiting autophagy with 3-methyladenine (3-MA) or by affecting the endosomal sorting complex required for transport (ESCRT) machinery, which has a role in endosome formation and protein sorting (Cipolat Mis et al., 2016). As so, autophagy acts in a coordinated manner with other pathways such as endocytosis (**Figure 3**). Functional endosomes are necessary for clearance of



**Figure 3** Autophagy pathway

Autophagy is an intracellular degradation pathway that delivers cytoplasmic constituents to the lysosome. It is an independent system from the endosomal-lysosomal pathway and the proteasome degradation pathway, although the three of them interact. There are three types of autophagy—macroautophagy, microautophagy, and chaperone-mediated autophagy—and the term “autophagy” usually indicates macroautophagy *Adapted from amsbio.com*

autophagosomes, and the disruption of endosomal proteins can lead to accumulation of polyubiquitinated TDP43 in FTD models (Wong and Cuervo, 2010).

The implications of autophagy seem to go beyond the local alteration of cellular homeostasis. A possible connection with aggregates spreading has been suggested as the kinetics of aggregate pathology progression suggests a non cellular-autonomous spreading: aggregates released by the cell through the autophagy pathway will be able to infect other cells. These aggregates will induce misfolding of proteins and aggregation in “healthy” cells in a prion-like manner. Furthermore, it has been shown that endocytosis is a major route of cellular entry for pathogenic forms of prion proteins (PrP<sup>Sc</sup>), and the coupling of autophagy and endocytosis can act as a bridge for aggregates propagation. Evidence in this direction is increasing for diseases like AD, HD, FTD and others (Eftekharzadeh et al., 2016; Ogen-Shtern et al., 2016) but further investigation is still needed to determine whether or not this mechanism is effectively responsible for propagation of aggregating proteins in neurodegenerative diseases.

### **1.3. p62, a link between autophagy and the ubiquitin proteasome system**

Autophagy is a key aspect of cellular homeostasis, and as previously seen, its failure causes various effects associated with neurodegeneration such as alterations in local axonal membrane trafficking and turnover (Komatsu et al., 2007). Its coordinated role with other protein degradation pathways allows for clearance of aggregating proteins that accumulate during disease. Several proteins link degradation pathway in a coordinated manner, of which, p62 has a particular role. p62, also known as sequestosome 1 (SQSTM1), is a multi-complex ubiquitin binding protein that recognizes toxic cellular waste and facilitates degradation through the UPS or through autophagosome-lysosome pathways (Bitto et al., 2014; Lin et al., 2013). The p62 protein is commonly found in inclusion bodies containing polyubiquitinated protein aggregates and expressed in stressful conditions such as oxygen radical stress and inhibition of proteasomal activity. It is also found in the brain of C9orf72 mutation carriers, under the form of TDP43 negative, ubiquitin positive inclusions. Of notice, mutations in the p62 gene can cause classical, adult onset Paget bone disease and FTD/ALS (Rea et al., 2014). It is a multifunctional protein capable of binding to ubiquitin at the surface of protein aggregates through an ubiquitin-associated (UBA) domain. Through this interaction, p62 brings autophagosome formation by linking with a LC3-interacting region (LIR) in a highly selective manner to ensure protein degradation (Birgisdottir et al., 2013; Pankiv et al., 2007);. The primary sequence of p62 has two nuclear localization signals (NLS) and one nuclear export signal (NES), allowing the protein to shuttle between nucleus and cytoplasm (Bitto et al., 2014). Another function of p62 has been described in amino acid sensing by its association with the mTORC1 complex subunit “Raptor”, and with several other Rab GTPases. The Rab protein family is implicated in regulation of multiple steps of membrane trafficking, including vesicle formation, vesicle movement along tubulin and actin networks, and membrane fusion. Through this interaction, p62 relays information from amino acid pool to the mTOR pathway, probably acting as a control mechanism to prevent excess autophagy and to avoid amino acid starvation (Bitto et al., 2014). Almost all inclusions of protein aggregates are p62 positive in autophagy deficient cells, suggesting that protein complexes aggregate in a p62 dependent manner (Komatsu et al., 2007). Furthermore, in several neurodegenerative diseases, protein aggregates co-localize with p62 (Bitto et al., 2014; Rea et al., 2014).

Contrarily to what was observed with modifications of the autophagy pathway, the ablation of p62 does not result in improvement or exacerbation of behaviors abnormalities,

such as tremor or abnormal limb claspings, in autophagy-deficient animals (Komatsu et al., 2007). Mutations of p62 that are associated with FTD/ALS locate close to or within the UBA domain and will lead to its defective recognition. These will directly impact upon protein-to-protein interactions, leading to an increase in NF- $\kappa$ B signaling pathways, through the interaction with TRAF6 (TNF associated factor 6) protein (Rea et al., 2014; Wooten et al., 2005). The NF- $\kappa$ B signaling pathway is important for neuronal differentiation and survival (Rea et al., 2014), and its role in ALS/FTD has been more broadly studied. Several genes causing ALS have been associated with altered NF- $\kappa$ B signaling in cellular and animal models, including *OPTN*, *VCP* and *TDP43*, and studies show upregulation of NF- $\kappa$ B expression in spinal motor neurons of ALS patients (Rea et al., 2014; Zhao et al., 2015). Interestingly, these genes have been shown to alter interaction with LIR regions, which are fundamental for autophagosome formation and growing evidence points towards the interplay between NF- $\kappa$ B signaling and the autophagy pathway.

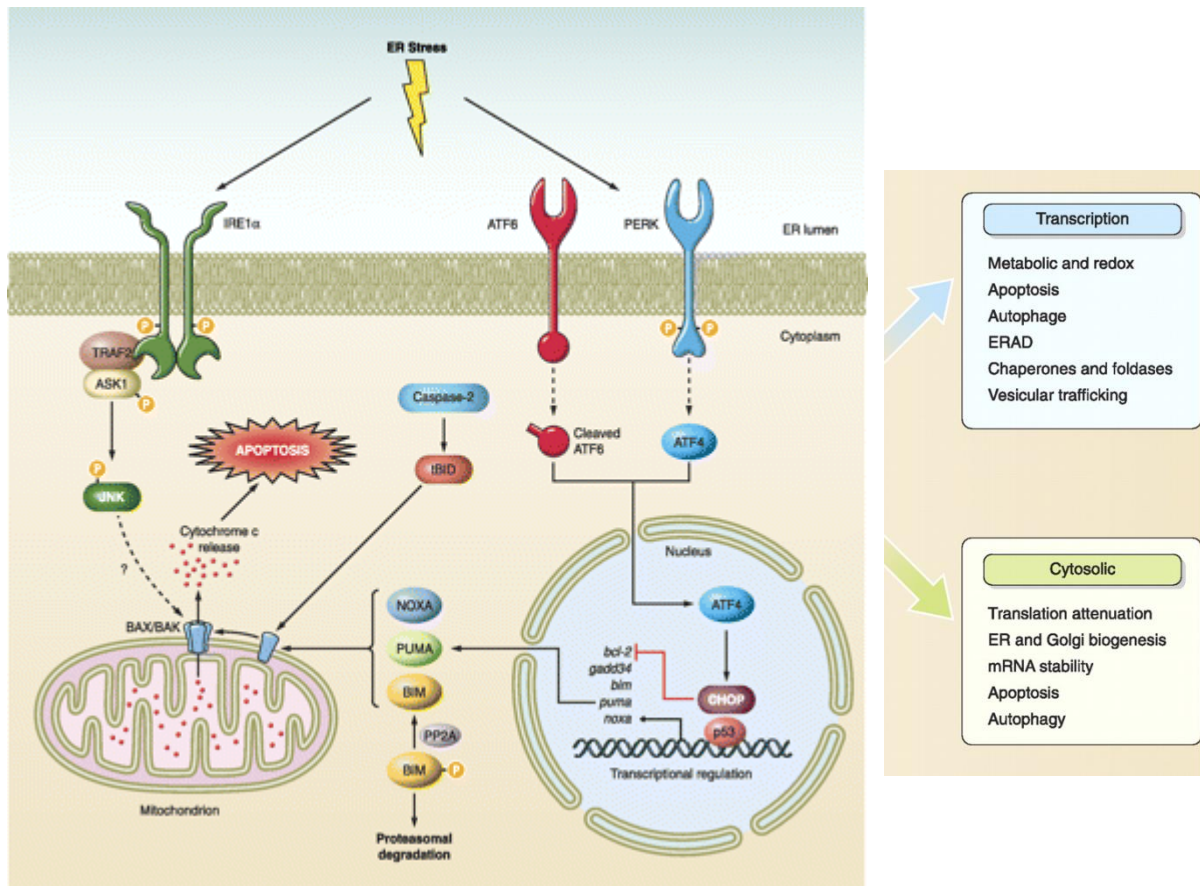
#### **1.4. Ubiquitin proteasome system and the unfolded protein response**

A cross talk exists between autophagy and the UPS, as blockage of autophagy results in massive accumulation of polyubiquitinated aggregates (Wong and Cuervo, 2010). This cross talk is mediated by p62, which is a putative substrate of both systems. The UPS is another protein degradation pathway that can be clogged by the presence of protein aggregates (Schmidt and Finley, 2014). The presence of ubiquitin within these inclusions suggests ineffective protein degradation by the proteasome. This inefficacy can arise from direct proteasome malfunction or from the inefficient targeting of misfolded proteins to the degrading pathway. *In vitro* studies have shown a conserved proteasome activity in the presence of aggregating proteins (Dantuma and Bott, 2014), but reduced proteasome activity has been reported in the *substantia nigra* of PD patients, and SOD1 aggregates, present in familial forms of ALS, have been found to sequester proteasome subunits, making it complex to assert between one hypothesis rather than the other (Ogen-Shtern et al., 2016).

Correctly folded proteins are transported to their final cellular localization by carrier proteins across cellular membranes such as the plasma membrane, endoplasmic reticulum and nuclear envelope via the secretory pathway. Proteins are also trafficked between membrane-bound organelles inside membrane vesicles (Rapoport, 2007). On the other hand, misfolded proteins are recognized and targeted for retro-translocation in the cytoplasm, where the UPS will be able to degrade them through a process known as ER-associated degradation (ERAD).

Inhibition of ERAD causes accumulation of misfolded proteins in the pericentriolar region of the cell. Accumulation of abnormally folded proteins at the ER engages an adaptive stress response (**Figure 4**). In the presence of this ER stress, the cell will activate the UPR mechanism to (1) diminish protein synthesis and to (2) up-regulate ER chaperons and (3) ERAD factors with the aim to reduce aggregate accumulation. If ER stress is not successfully diminished, the UPR will initiate an (4) apoptotic phase culminating in cellular death (**Figure 4**) (Hetz et al., 2011; Schmidt and Finley, 2014). Aggregating proteins in neurodegenerative diseases, such as tau or  $\alpha$ -synuclein, are capable of inducing the UPR. In AD, Tau and A $\beta$  soluble oligomers are likely to inhibit ERAD leading to UPR activation and in PD,  $\alpha$ -synuclein is capable of inhibiting autophagy factor 6 (ATF6) trafficking to the Golgi and thus causing UPR activation (Ogen-Shtern et al., 2016; Schmidt and Finley, 2014).

Yet, whether autophagy and proteasome degradation targets similar normal or misfolded proteins is a question that remains to be answered. Furthermore, as these two systems crosstalk between each other, it is often difficult to determine which of these processes, if any, is associated with the initial disease mechanisms. It is plausible that these two systems act in a simultaneous and complementary manner, particularly when aberrant proteins overwhelm the disposal capacity of the proteasome.



**Figure 4** ER stress-mediated apoptosis and the Unfolded Protein Response (UPR)

Under prolonged or chronic ER stress due to accumulation of abnormally folded proteins, activation of an apoptotic response will be engaged. There are three major ER-UPR stress sensors, IRE1 $\alpha$ , PERK, and ATF6, which transduce information about the folding status of protein in the ER to the cytosol and the nucleus to restore folding capacity. Activation of IRE1 $\alpha$  controls selective mRNA decay and also leads to the processing of the mRNA encoding XBP1, a transcription factor that upregulates many essential UPR. Active IRE1 $\alpha$  also regulates stress responses mediated by JNK, ERK, and NF $\kappa$ B. Activation of PERK decreases the general protein synthesis rate through phosphorylation of the initiation factor eIF2 $\alpha$ . eIF2 $\alpha$  phosphorylation will increase the translation of the ATF4 mRNA, which will induce the expression of genes involved in amino acid metabolism, autophagy, antioxidant responses, and apoptosis. Activation of the pro-apoptotic BAX and BAK at the mitochondria is a key step in the induction of apoptosis, leading to the release of cytochrome *c* and activation of downstream caspases. The transcription factor CHOP activated by IRE1 $\alpha$ , PERK, and ATF6 will downregulate the anti-apoptotic protein BCL-2. In addition, active IRE1 $\alpha$  binding to TRAF2 will lead to the activation of the pro-apoptotic kinases JNK and ASK. *Adapted from Hetz et al. (2011)*

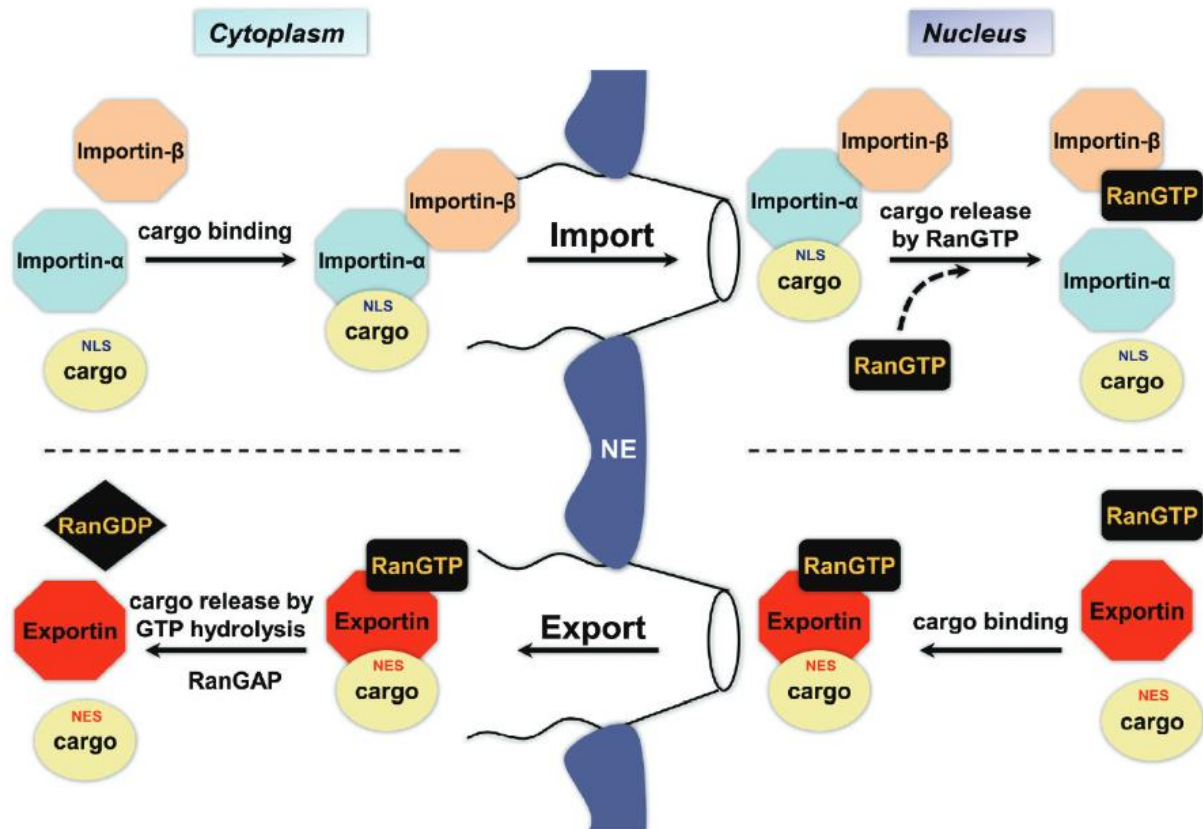


### 1.5. Nucleocytoplasmic shuttling dysfunction

Several mechanisms could explain altered subcellular distribution of proteins, but growing evidence has placed disruption in nucleocytoplasmic transport (NCT) as central to the impairment of neuronal functions leading to neurodegeneration. Aggregation in the cytoplasm is able to interfere with nucleocytoplasmic shuttling and RNA transport (Woerner et al., 2015). Thus, the study of mechanisms altering NCT may play a significant role in understanding pathogenesis of neurodegenerative diseases.

Small proteins (<40kDa) can passively diffuse through the nuclear envelope but larger proteins will require active transport to be carried out between the nucleus and the cytoplasm with the assistance of importins for nuclear import and exportins for nuclear export (**Figure 5**). Molecules are able to enter and exit the nucleus through the nuclear pore complex (NPC). This large complex is composed of over 30-50 protein subunits called nucleoporins (Nups), each of them composed by several hundred individual protein-molecules (Patel and Chu, 2011; Rout et al., 2000). With aging, the composition of the nuclear pore can be altered and become “leakier” and in pathological conditions, the nuclear contour itself can be modified. In ALS and in PD models, staining of NPC nups-p62 protein has shown irregularities in the nuclear contour that present ruffled abnormal edges. In AD cases, Nrf2 protein, which is also implicated in NPC conformation, presents cytoplasmic mislocalization rather than the normal nuclear one (Patel and Chu, 2011). As neurons are polarized cells, molecule shuttle along the axon and the soma is a highly regulated process in order to maintain correct functional structure. Lesion studies have shown that neurons can activate a high response rate to damage, in order to avoid alterations. For example, in the case of nerve lesion, local translation of axonal mRNA increases importin  $\beta$  protein levels allowing for the formation of units that transport proteins backward and forward to ensure repairing (Patel and Chu, 2011). This shuttling will activate signals such as phosphorylation or acetylation that in turn, can alter the nuclear to cytoplasmic ratios of several proteins. Oxidative stress and protein aggregates are yet other stimuli that will lead to imbalance in nuclear to cytoplasmic ratio of proteins, but also in nucleoporins and other proteins of the nuclear import and export machinery (Patel and Chu, 2011; Woerner et al., 2015).

From this point, protein aggregates are of particular interest, as identical proteins with different targeting sequences, either to the nucleus or the cytoplasm can form distinct biochemical aggregates that differ in toxicity. Such is the case with expression of cytoplasmic-prone aggregating proteins that will cause altered distribution of the NPC



**Figure 5** Schematic representation of nucleocytoplasmic shuttling

Import of NLS-containing cargo protein through the NPC occurs via Importin  $\alpha$ /importin  $\beta$  heterodimer. In the nucleus, RanGTP binds to importin  $\beta$  to induce release of cargo protein. Importin  $\beta$  will then be transported to the cytoplasm with the exportin CAS (cellular apoptosis susceptibility protein) to be recycled upon GTP hydrolysis. Export of NES-containing cargo protein occurs with the interaction of exportins. Protein release to the cytoplasm will occur upon GTP hydrolysis. NTF2 (nuclear transport factor 2) will recycle RanGDP back to the nucleus. Ran regulators, RanGAP in the cytoplasm and RanGEF in the nucleus, highly preserve RanGDP/RanGTP gradient, as it is fundamental for determining the sense of NCT. NE = Nuclear envelop *Adapted from Patel and Chu (2011)*

proteins, interfering with import and export proteins and mRNA transportation. Nucleus-prone aggregating proteins, on the other hand, will have no effect in NPC protein distribution or function, as was shown in *in vitro* models of protein aggregation (Woerner et al., 2015). Mechanisms through which cytoplasmic protein aggregates exert this toxicity in the NPC remain elusive. The toxic effect could pass through aberrant interactions with other proteins, resulting in their functional impairment and sequestration, especially since cytoplasmic aggregates bind splicing factors, RBPs and other proteins involved in the nuclear transport (Woerner et al., 2015). Nuclear aggregates, in the other hand, bind mainly RBPs, probably due to their intrinsic properties which make them more prone these association or to their

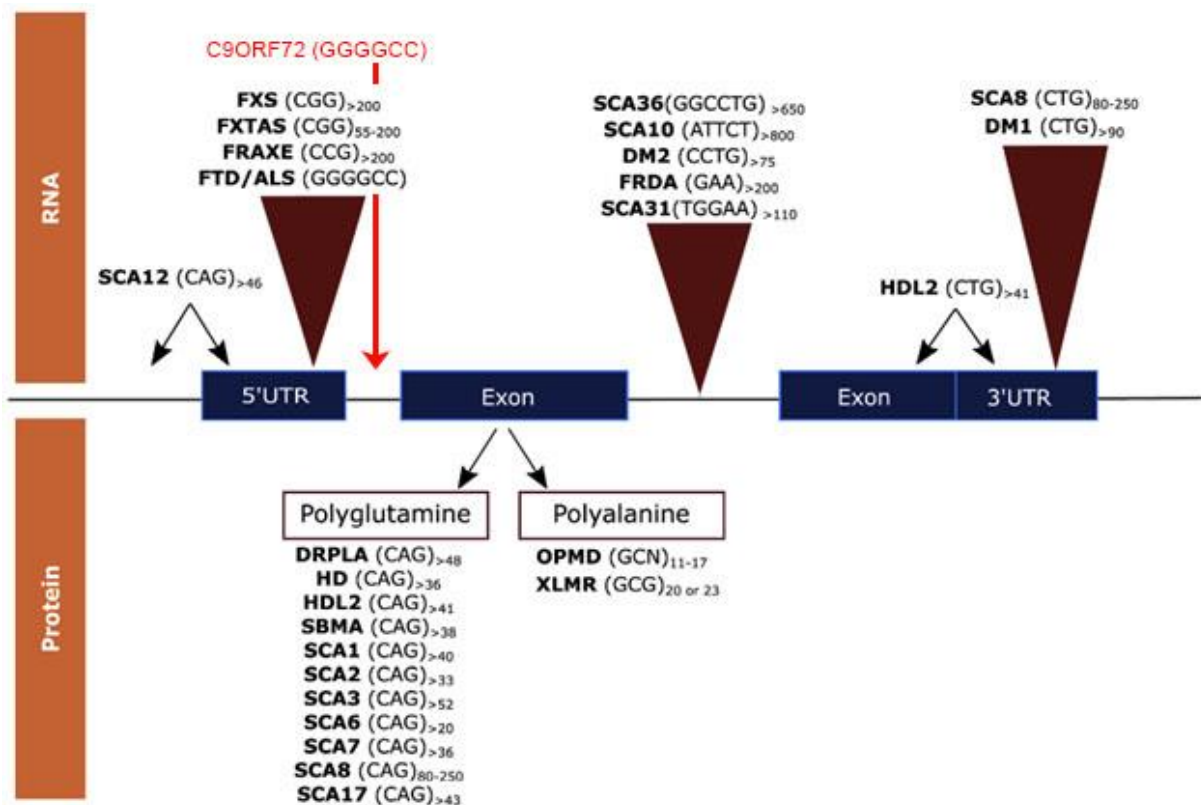
interaction with specific nuclear proteins such as nucleophosmin 1 rather than cytoplasmic proteins (Woerner et al., 2015).

Nuclear export is a less well-known process and it mainly concerns RNA molecules linked to ribonucleoproteins (RNP), such as tRNAs, ribosomal subunits and mRNAs. Emerging evidence has given a role to nuclear export in the regulation of microRNA (miRNAs) biogenesis process (Muqbil et al., 2013). miRNAs are short non-coding RNAs of 18 to 25 nucleotides in length that can regulate the expression of a myriad of genes and their implications will be explored in the following section.

## **2. RNA metabolism alteration in neurological disease**

Regulation of RNA metabolism in neurons is highly elaborated, involving compartmentalized expression through long distance mRNA trafficking, local translation and alternative splicing (Gao and Taylor, 2014). Interest in this pathway rose after the identification of altered RNA metabolism in abnormal microsatellite expansions cases. The identification, in 1991 that the CGG expansion in the 5' non coding region of the FMR1 gene was responsible of Fragile-X syndrome (FXTAS) opened the door to the discovery of numerous neurological and neuromuscular diseases caused by repeat expansions (Fu et al. 1991, Oberle et al. 1991; Verkek et al. 1991). Over 40 neurological, neurodegenerative or neuromuscular diseases are explained by these type of mutations, and they include some of the most common inherited disorders such as HD, spinocerebellar ataxias (SCAs), and FTD/ALS caused by an expanded repeat in *C9orf72* (c9FTD/ALS) (DeJesus-Hernandez et al., 2011; Loureiro et al., 2016; Maizels, 2015; Ranum and Cooper, 2006; Renton et al., 2011). Typically, the number of repeats in non-affected individuals is lower than 30, but it is higher that 40-50 in patients, and may exceed 1000 repeats in some cases (Sicot and Gomes-Pereira, 2013). Some of these disorders present genetic anticipation, as genetically unstable repeats can undergo further expansion during intergenerational transmission (Mirkin, 2007), whereas for some others, the type of inheritance has not been yet identified. More than 20 years later, our understanding of these “microsatellite-expansion diseases” has advanced, but has also risen questions concerning how these expansions are be able to result in disease. I will not refer to microsatellites present in the genome that are not disease-causing expansions. In this chapter, I will describe findings in the field of neurodegenerative diseases with a focus on the neuropathological mechanisms driven by RNA toxicity, pointing to aspects linked to the hexanucleotide repeat expansion in c9FTD/ALS.

Microsatellite-expansion disorders are caused by a repetitive DNA tract, in which a DNA motif ranging in length from 2 to 6 nucleotides pairs is bi-directionally transcribed producing toxic mRNA products (Cleary and Ranum, 2014; Ranum and Day, 2002). Mutations in microsatellite-expansion disorders can be located either in the coding region or in the noncoding region of affected genes (**Figure 6**). In coding regions, repetitive tracts causing either polyglutamine or polyalanine diseases are the sole type of expansion being expressed, leading to the expression of expanded RNA and proteins. In non-coding regions, a variety of repeats can be observed, ranging from trinucleotide up to hexanucleotide expansions repeats (Loureiro et al., 2016). In the case of non-coding repeats DNA expansion mutations be can also expressed at the protein level in an ATG-independent manner through RAN (repeat associated non-ATG) translation (Cleary and Ranum, 2014). Normal alleles of genes associated with expansions contain stabilizing interruptions of the repeat along the genomic sequence. When these interruptions are deleted during the replication or transcription processes, the number of repeats will exceed what is consider a normal threshold becoming pathogenic and prone to expansions (Mirkin, 2007). This instability is what makes repeat expansions mutations to be known as dynamic mutations leading to repeat size increase through generations. Repeat mutations can also occur in somatic tissues resulting in a somatic mosaicism, which will contribute to the phenotypic variability and progression of disease over time (Sicot and Gomes-Pereira, 2013).



**Figure 6** Coding and non-coding expansions associated to neurodegenerative diseases

Schematic representation showing the localization of repeat expansions associated with neurodegenerative diseases. Repetitive tracts can be located either in coding (upper part) and non-coding gene (lower part) regions. *Adapted from Loureiro et al. (2016)*

## 2.1. Molecular mechanisms leading to repeat expansion formation

Models for repeat expansions assume that these alterations will mostly occur during DNA replication due to the fact that rapid accumulation of intermediate repetitive DNA can take place during the progression of the replication fork (Mirkin, 2007). DNA unraveling during repair and recombination processes will also lead to the formation of mismatched structures that can eventually result in the formation of repeat expansions. Such is the case in a transgenic mice model of HD, in which single base excision repair mechanism caused the formation of hairpin structures that resulted in a final integration of a repeat expansion structure (Mirkin, 2007). Furthermore, expandable repeats are also capable to induce large chromosomal rearrangements (Bacolla et al., 2006).

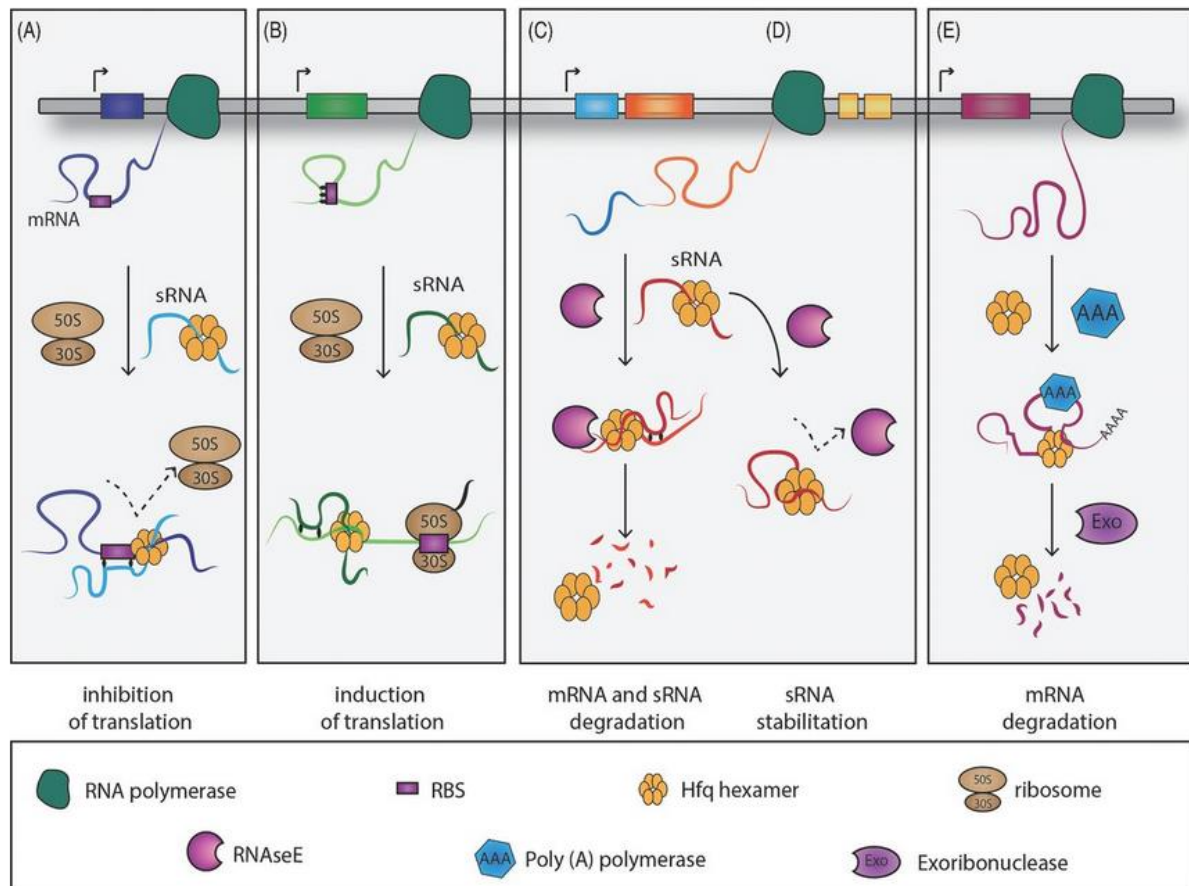
Expansions of G-quadruplex are common among repeat expansion diseases. These G-quadruplex structures can form thermodynamically stable DNA or RNA structures. Several

factors have been shown to bind G-quadruplex, as have been domains like RRM/RBD and RGG motifs found in different nuclear RNA binding proteins (Maizels, 2015). Consequently, GGGG motifs may influence genomic stability, transcription and translation, and they have been shown to play a role in several cancers and in FXTAS and c9FTD/ALS.

## **2.2. Molecular mechanisms leading to repeat-mediated toxicity in the case of non-coding expansions**

The sequence and the location of the repeat expansion within a gene will have specific consequences on human disease onset and development. Transcription of non-coding DNA repeats will lead to the production of expanded RNA transcripts. These transcripts will be the cause of “RNA toxicity” which will induce cellular death through several non exclusive pathways, including RBPs sequestration and stress-induced cellular death (Maizels, 2015; Mirkin, 2007). Evidence of this RNA toxicity has now been found in several neurodegenerative diseases, such as FXTAS, SCA8, 10, 12, and 36 (Sicot and Gomes-Pereira, 2013). In DM1 and 2 for example, expansion of a GTG trinucleotide and of a CCTG tetranucleotide repeat expansion respectively in two unrelated genes, will form RNA transcripts that are retained to the nucleus forming RNA aggregates (or foci) (Ranum and Day, 2002; Sicot and Gomes-Pereira, 2013). The abnormal interaction between these RNA foci and RBPs will contribute to cell dysfunction and disease phenotype. In the case of DM1 and DM2, two families of splicing regulators, MBNL (muscleblind-like) and CELF (CUGBP, Elav-like family), colocalized in RNA foci suggesting RNA toxicity via the sequestration of RBPs as a possible pathomechanism. This sequestration leads to functional depletion and abnormal localization of the captured protein and splicing abnormalities, as was observed in DM1 and DM2 cases (Charlet-B. et al., 2002). Microarrays studies in muscle biopsies of these pathological cases showed abnormal gene expression that could be explained in 60-70% fold by loss of MBNL1, and flies models showed that loss of MBNL2 would cause relocalization of MBNL targets (Wang et al., 2012). These observations suggest that affecting splicing regulators will not only affect the alternative splicing of target transcripts, but also gene expression and RNA and protein localization.

More recently, the notion that interfering with or sequestering RBPs leads to neurodegeneration has been shaped by discoveries in the ALS and FTD. Mutations in heterogeneous nuclear ribonucleoproteins (hnRNPs) A1 and A2/B1 are causative for ALS (Kim et al., 2013). hnRNPs correspond to a large family of RNA-RBPs that contribute to



**Figure 7** Roles of RBPs in RNA metabolism

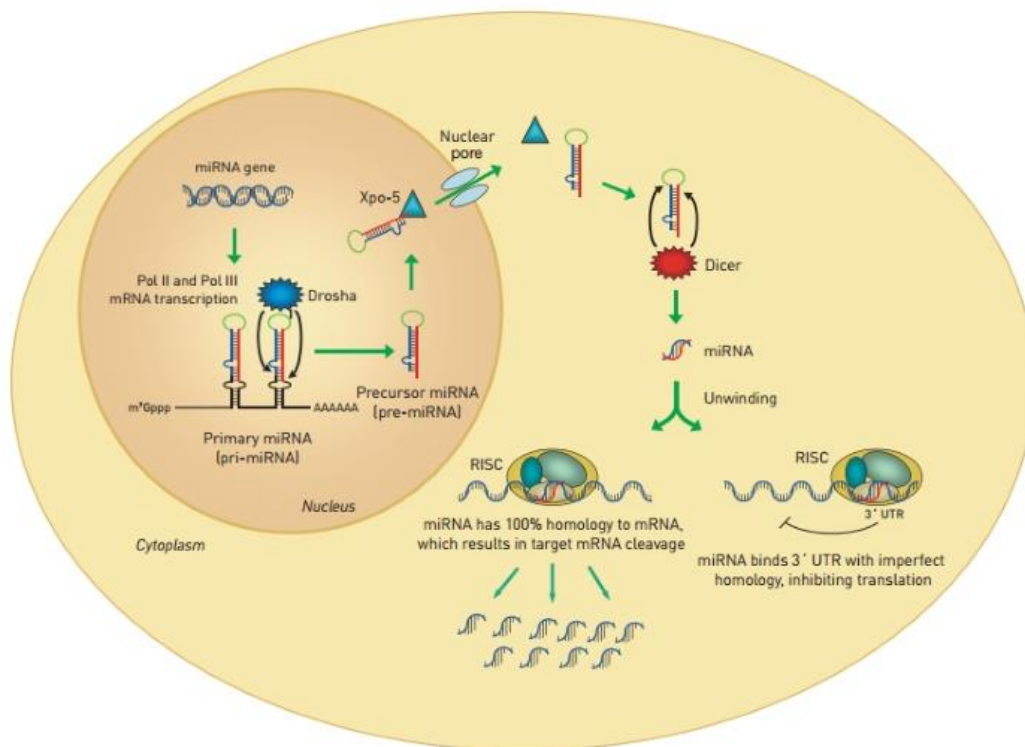
Regulation of RNA metabolism is ensured by RBPs by several mechanisms including (A) inhibition at the transcriptional level of the targeted mRNA, (B) induction of specific translation, (C) modulation of RNA turnover and (D) protection of sRNA cleavage, and finally (E) facilitation of targeted polyadenylation of mRNA. *Adapted from Olivia et al. (2015)*

multiple aspects of RNA and DNA metabolism including alternative splicing, mRNA stabilization, and transcriptional and translational regulation (**Figure 7**). Interestingly, hnRNP A1 and A2/B1 can be found sequestered in cytosolic stress granules together with Fused in Sarcoma (FUS) and TDP-43. As TDP43, FUS is also an RBP, and both proteins share striking structural and functional similarities (Lagier-Tourenne et al., 2010). FUS is also known as hnRNP P2, and mutations in its C-terminal NLS region are causative for FTD/ALS (Waibel et al., 2010).

RNA foci are constantly forming and disaggregating (Sicot and Gomes-Pereira, 2013) allowing for RNA transcripts to be translated by RAN translation. The generation of toxic neuropeptides from expansion by RAN translation might also end in cellular toxicity and death (Cleary and Ranum, 2014; Loureiro et al., 2016; Mizielinska and Isaacs, 2014). RAN translation has been widely studied in c9FTD/ALS. Patients with the C9orf72 repeat

expansion present expanded alleles ranging from 30 up to 4000 repeats, whereas in healthy individuals, repeat number is lower than 30 repeats (Beck et al., 2013; Loureiro et al., 2016). In this case, the GGGGCC transcript will be able to escape the nucleus and be translated into aggregating dipeptide-repeat proteins (DPRs) in the cytoplasm forming ubiquitin and p62 positive inclusions (Haeusler et al., 2014; Mackenzie et al., 2013). These protein aggregates have been found in patient brains of c9FTD/ALS, but also in other neurodegenerative diseases caused by non-coding expansion, as is the case for example of SCA8, DM1, FXTAS and (Cleary and Ranum, 2013).

Finally, as previously introduced in NCT, miRNA are involved in neurodegenerative diseases, and particularly in FTD/ALS. miRNAs are noncoding RNAs that regulate gene expression through 3' Untranslated Regions (3'UTRs). Primary miRNAs are initially cleaved



**Figure 8** RNA interference and the RISC complex

RNA interference (RNAi) is a post-transcriptional process triggered by the introduction of double-stranded RNA (dsRNA) which leads to gene silencing in a sequence-specific manner. Long dsRNAs are cleaved by the RNase III family member, Dicer, into 19-23 nucleotides fragments called mi-RNAs. The endonuclease Argonaute 2 (Ago 2) catalyzes the unwinding of the miRNA duplex. Once unwound, the guide strand is incorporated into the RISC, while the passenger strand is released. RISC uses the guide strand to find the mRNA that has a complementary sequence leading to the endonucleolytic cleavage of the target mRNA. *Adapted from ThermFische.com*



by Drosha to be subsequently exported to the cytoplasm by exportin 5 (XPO5) where they will be processed by the Dicer complex at the level of the RNA induced silencing complexes (RISC) to result in mature miRNAs (**Figure 8**) (Eitan and Hornstein, 2016; Muqbil et al., 2013). Mature miRNAs will target numerous mRNAs to regulate the levels of several genes in the same pathway in a coordinate manner (Gascon and Gao, 2014). Emerging roles for these mature mRNAs arise and even though their implications in neurodegeneration have not been fully determined, more and more data showing deregulation of specific miRNAs in independent cohorts of patients argues in favor of their crucial role in pathogenesis (Gascon and Gao, 2014; J. Y. Tan et al., 2014). In myotonic dystrophy 1 it was shown that transcription of sense and anti-sense mutated expansions led to alteration of RNA interference (RNAi) mechanisms (Mirkin, 2007), and in FTD/ALS, miR-134 and miR-34 are highly dysregulate. Reminiscent evidence has suggested that mutated RNA hairpins formed of repeat expansions in the case of c9FTD/ALS could short circuit miRNA biogenesis, possibly explaining their deregulation (Malinina, 2005; Mirkin, 2007). Alternatively, miRNAs might simply be altered due to TDP43 aggregation in these cases altering its function in miRNA biogenesis.

From this initial presentation of mechanisms involved in neurodegenerative diseases, it is possible to discern how different pathways interlace. In the center of both mechanisms, protein aggregation and RNA toxicity, is the example of TDP43 and FUS pathology, reminiscent of FTD and ALS. Understanding mechanisms that lead to toxicity through these pathways will help elucidate major pathogenic pathways in several other neurodegenerative diseases. During the next chapter, I will present clinical and pathological presentations of both FTD and ALS.

### **III. Frontotemporal degeneration and amyotrophic lateral sclerosis: two ends of a same disease spectrum**

At first glance, clinicians might not think that a paralytic disease caused by degeneration of upper and lower motor neurons could be linked mechanistically to a dementia presenting progressive changes in personality and language. Yet, frontotemporal dementia (FTD) and amyotrophic lateral sclerosis (ALS) frequently occur in the same family and/or in the same patients. Identification of mutations in fused in sarcoma (FUS), Tar-DNA-binding protein (TARDBP) and in Chromosome 9 open reading frame 72 (C9orf72) causing both FTD and ALS represent a strong molecular link between these two diseases.

In this chapter, I will present relevant information concerning the phenotypical continuum of FTD-ALS, briefly describing major clinical descriptions and highlighting major physiopathological hypothesis of both FTD and ALS. I will then present current knowledge of the link between these two related diseases.

#### **1. Features of frontotemporal dementia**

##### **1.1. Epidemiology and clinical features**

FTD denotes a group of heterogeneous sporadic and familial neurodegenerative disorders characterized by progressive neuronal loss predominantly involving the frontal and temporal lobes. It is considered the second most common cause of early-onset dementia after AD (Bang et al., 2015), and it constitutes over 20% of neurodegenerative dementias (Lee, 2005). Clinically, FTD is mainly a behavioral disease with significant changes in social and personal behavior, apathy, blunting of emotions, hyper-orality and dietary changes, and deficits in both expressive and receptive language.

It was in 1892, that Arnold Pick firstly reported a patient with progressive aphasia and anterior temporal atrophy. Pick's disease was considered the prototypical of FTD. However, this idea has been deflected in recent years after identifying that a mutation can produce diverse clinical phenotypes, ranging from movement disorders to memory dysfunctions (Lee, 2005). FTD is now considered as a larger spectrum of syndromes.

Three subtypes are used to describe FTD according to clinical observations and lesion sites: (1) behavioral variant FTD (bvFTD) presenting a fronto-lateral symmetric atrophy; (2) semantic variant primary progressive aphasia (svPPA) with lesions mainly in the anterior left temporal lobe; and (3) non-fluent variant primary progressive aphasia (nfvPPA) with lesions

	BV-FTD	SV-PPA	NFV-PPA
<b>Social symptoms</b>			
Disinhibition, inappropriate or offensive behaviour, excessive jocularity, exaggerated emotional display, impulsivity, inappropriate sexual remarks, lack of embarrassment	73-98%	59%	18%
Loss of empathy, lack of emotional insight, social coldness	49-78%	28-49%	18%
Selfishness, disregard for others' feelings	83-89%	91%	No
Aggression	25-61%	28-64%	12%
Personal neglect, neglect of hygiene	83-92%	64%	No
Strange manner of dressing	Yes	Right-sided cases	No
Personality changes	Sometimes	Sometimes	Rarely
<b>Emotional symptoms</b>			
Apathy, low motivation, asponaneity, decreased initiation of behaviour	54-96%	18-47%	41%
Depression, emotional detachment	Common	44%	Sometimes depression
Irritability	33%	50%	47%
Anxiety, social avoidance	No	Not usually	Yes
Exaggerated emotional display	33-39%	55%	Rarely
<b>Eating and oral behaviours</b>			
Overeating, gluttony	61-83%	36%	Rarely
Reduced selectivity, indiscriminate eating	41-55%	9%	Rarely
Increased selectivity, food fads	8-22%	55%	Rarely
Preference for sweet foods	25-56%	36%	Rarely
Preference for savoury foods	0%	9%	Rarely
Hyperorality	0-22%	18%	Rarely
<b>Repetitive or compulsive behaviours</b>			
Behavioural stereotypes	95%	72%	24%
Compulsive behaviours	5-15%	60-80%	Rarely
Word obsessions, repetitious use of verbal expressions	Sometimes	Often	Rarely
<b>Sensory symptoms</b>			
Reduced pain response	39-45%	27%	Rarely
Increased pain response, increased heat/cold response	0-33%	45-55%	Rarely
Incontinence	Sometimes	Sometimes	Rarely
<b>Motor symptoms</b>			
Motor neuron disease	15%	Rarely	Rarely
Parkinsonism	18-20%	3%	6%
Progressive supranuclear palsy syndrome	Rarely	Not known	Sometimes
Corticobasal syndrome	Rarely	Not known	Sometimes
<b>Executive symptoms</b>			
Mental rigidity, inflexibility	41%	38%	12%
Inattention, disorganisation	Sometimes	Sometimes	Rarely
Loss of sensitivity to negative consequences, bad financial judgment	Sometimes	Sometimes	Rarely
<b>Language symptoms</b>			
Impaired word finding	45%	84%	71%
Apraxia of speech	5%	6%	94%
Agrammatism	0%	0%	53%
Impaired confrontation naming	30%	97%	71%
Impaired single-word comprehension	5%	84%	18%
Object agnosia	2%	34%	0%
Phonological errors	2%	9%	71%
Word repetition errors	0%	0%	53%
Sentence repetition errors	2%	6%	35%
Sentence comprehension errors	23%	72%	88%
Surface dyslexia	11%	81%	71%
<b>Neuropsychiatric symptoms</b>			
Delusions	14%	9%	6%
Hallucinations	20%	6%	6%
<b>Other symptoms</b>			
Sleep disorders	Sometimes	Rarely	Rarely
Restlessness	Sometimes	Sometimes	Rarely
Prosopagnosia	3%	47%	0%
Decreased libido	Sometimes	Sometimes	Rarely
Episodic memory deficits	Rarely	Rarely	Rarely

BV-FTD=behavioural-variant frontotemporal dementia. SV=semantic variant. NFV= non-fluent variant. PPA=primary progressive aphasia.

**Figure 9** Clinical symptoms of FTD

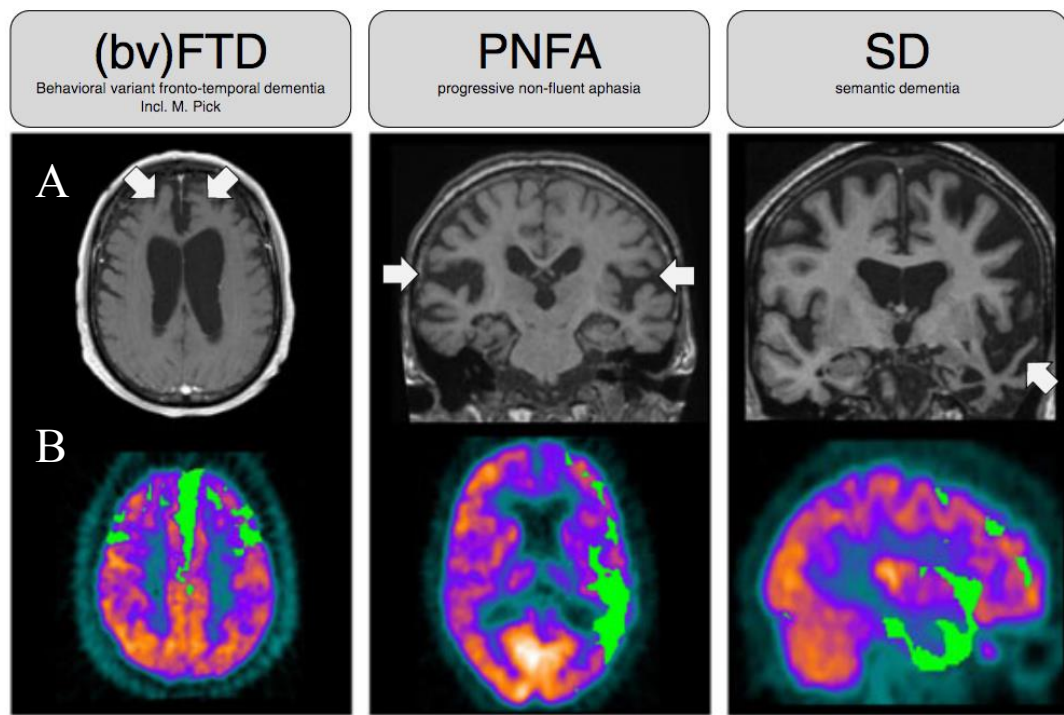
Presentation of common FTD symptoms and percentage of appearance in bvFTD, SV-PAA and NFV-PAA *From Bang et al. 2015*

in the left temporal lobe (Bang et al., 2015). Even though these groups allow for a better classification of clinical symptoms, patients exhibit a wide range of specific cognitive and behavioral deficits (**Figure 9**). These include impairment of executive functioning that interfere with strategic processing and working memory, lack of initiative and poor attention, disinhibition, hypersexuality, hyperorality behaviors and non-fluent speech that progresses to mutism. FTD-patients can also develop extra-pyramidal signs such as rigidity and gait disturbances that may be accompanied by a resting tremor (Lee, 2005). As disease progresses, symptoms can converge as the degeneration becomes more diffuse affecting larger areas of the brain (Bang et al., 2015).

## 1.2. Diagnosis

Differential diagnosis of FTD is done according to clinical observations examining the progression of corresponding behavioral deficits, familial history and neuroimaging. Onset generally occurs in individuals less than 60 years of age, but late onset cases are not uncommon (Bang et al., 2015). It affects equally men and women. As behaviors are so widely affected in FTD, misdiagnosis might happen with disorders such as obsessive-compulsive disorder, depression, or even bipolar disorders and schizophrenia (Bang et al., 2015).

The expected clinical course of the neurodegeneration is obviously progressive, but the natural history of FTD, reflective of the significant variability in phenotypic expression, makes prognosis for patients and family members highly challenging. It is not uncommon for clinicians to see fluctuation in disease progression in a random manner (Bang et al., 2015). Volumetric analysis with MRI can provide quantitative measurements of tissue loss, and atrophy over time revealing frontal and/or anterior lobe atrophy in late stages of the disease (**Figure 10A**) (Kelley and El-Khoury, 2016). Furthermore, functional connectivity network mapping with MRI has shown that the salience network is specifically affected in bvFTD (Zhou and Seeley, 2014). Anchored in the anterior insula, the salience network includes the anterior-cingulate cortex, ventral striatum, amygdala, dorsomedial thalamus, hypothalamus and brainstem nuclei including periaqueductal gray, *substantia nigra* and ventral tegmental area. It is a multimodal information processing hub identifying and relying relevant (salient) stimuli (Seeley et al., 2007). On the other hand, positron emission tomography (PET) scans allow to understand metabolic abnormalities as diseases progresses. Classically, FTD patients present a distinctive pattern of hypometabolism in frontal, anterior cingulate, and anterior temporal regions (**Figure 10B**) (Foster et al., 2005). Diffusion tensor imaging also helps in



**Figure 10** Overview of FTD alterations – Brain images

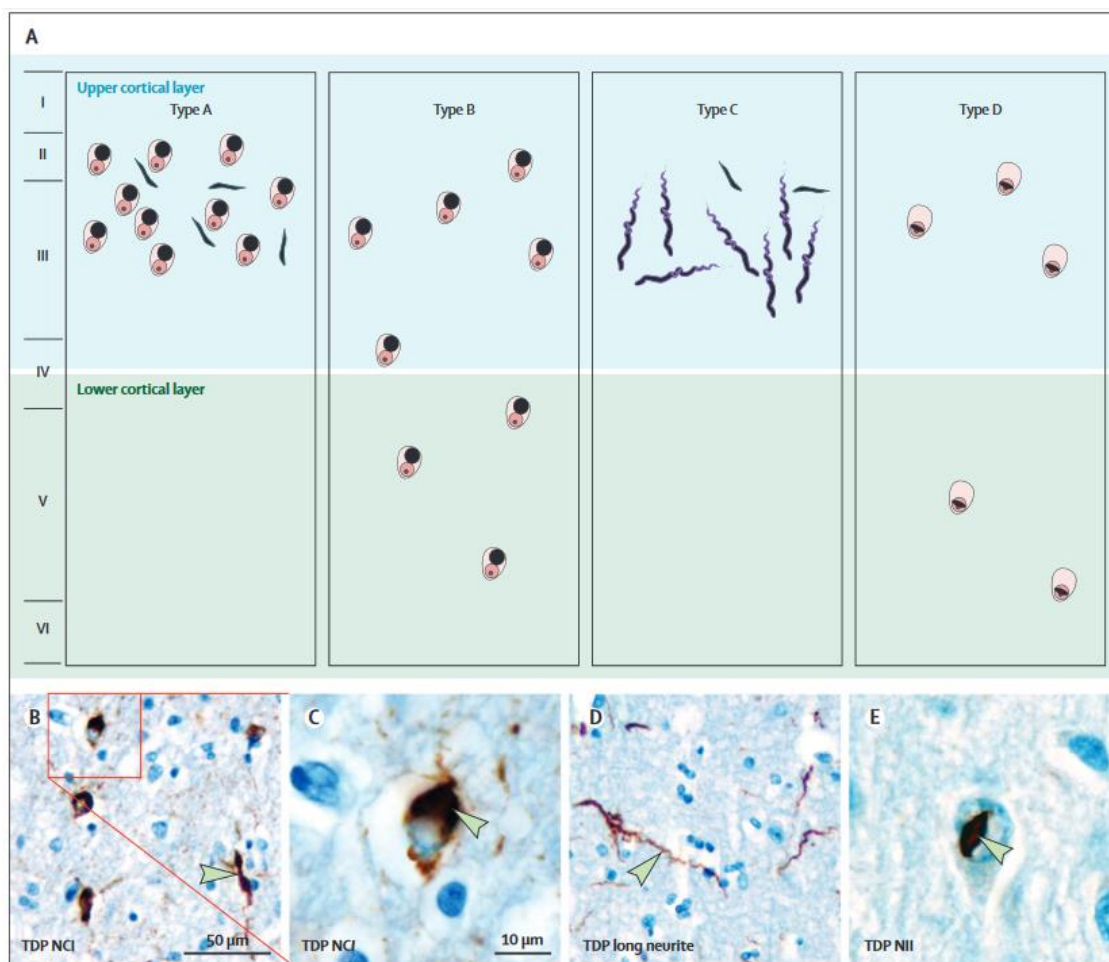
FTD patients brain images in MRI (A) and PET (B) images showing a frontal atrophy and a hypometabolism with anterior to posterior gradient. *Adapted from Haller et al. (2013)*

diagnosis by showing a widespread white-matter degeneration in FTD patients (Bang et al., 2015). As research advances, great improvement in differential diagnosis has been made; however, as variations are highly common between patients, further research is needed to understand the relationship between genetic factors, neuropathology and brain lesions. The next frontier is in molecular imaging, with various forms of amyloid and tau tracers that will allow differentiating between AD, non-AD tauopathies, and tau-negative dementias (Engler et al., 2008; Johnson et al., 2013; Whitwell et al., 2012; Zhang, 2015).

### 1.3. Neuropathology

FTD patients present large neuropathology heterogeneity with a relatively selective form in the frontal and temporal lobes. FTD is characterized by neuronal loss, gliosis, and microvacuolar changes of frontal, temporal lobes but also in anterior cingulate and insular cortices. Patients have been found to develop abnormal intracellular accumulation of disease specific proteins, which has allowed the classification of subtypes of FTD (Mackenzie et al., 2009; Rademakers et al., 2012). Approximately 50-60% of inclusions are of phosphorylated TDP-43, 30% develop accumulation of hyperphosphorylated and insoluble forms of tau, and inclusions of FUS protein have been observed in 10% of cases (Burrell et al., 2016; Lee,

2005; Rademakers et al., 2012). Pathological depositions initiate in the anterior cingulate cortex, and direct to the fronto-insular cortex, the orbitofrontal cortex, and the cingulate-frontal transitional zones in early stages. In later stages, pathology will evolve towards brainstem and spinal motor regions involving finally the visual cortex and most of the brain. Initial regions are constituted of Von Economo-neurons (VEN) and fork cells of layer 5 of the cortex, which degenerate very early in bvFTD (Bang et al., 2015; Burrell et al., 2016; Seeley et al., 2006). VEN are large bipolar neurons representing 5% of neurons present in layer V of the cingulate anterior cortex in humans and apes (Seeley et al., 2006). Their anatomical localization and projections in several brain areas suggest an important role in complex social interactions, particularly self-consciousness and social emotion (Morel et al., 2013).

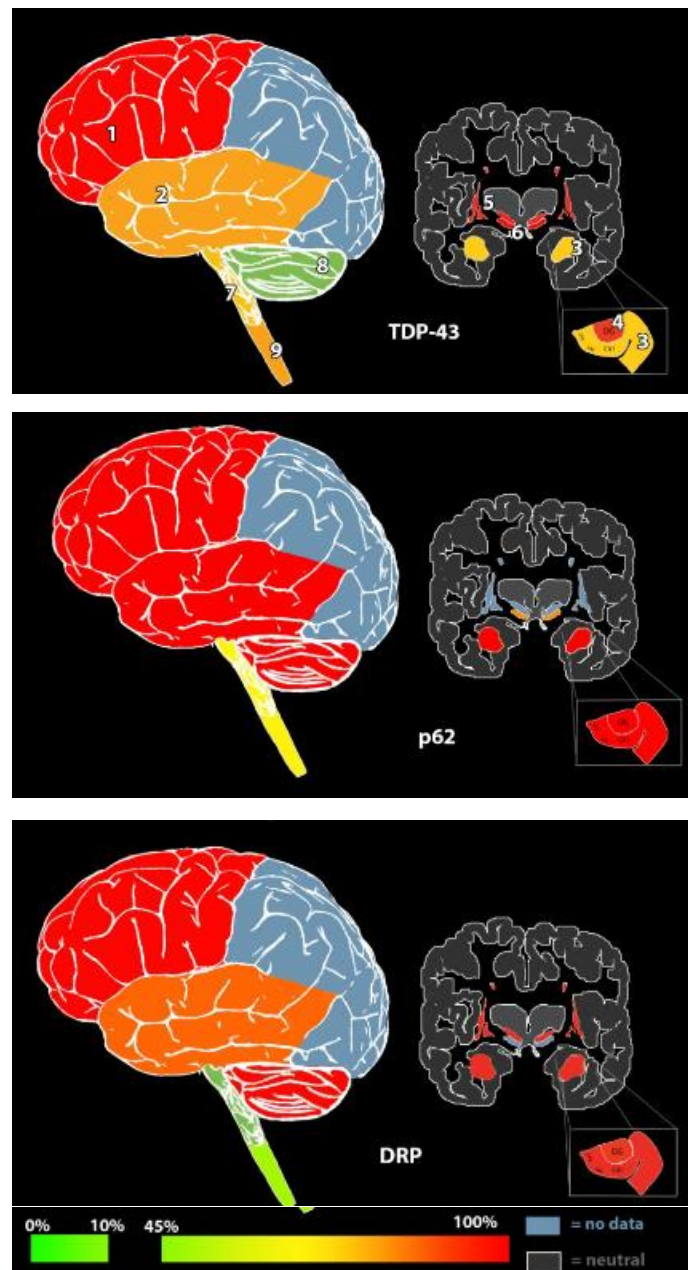


**Figure 11** Pathological inclusions of TDP43 found in FTD and ALS

(A) Schematic distribution of TDP43 inclusions in through the cortical layers as found in patients. (B) Neuronal cytoplasmic inclusions type A in upper cortical layers & (C) type B in lower cortical layers. (D) Long neurites staining in type C. (E) Intranuclear inclusions in type D. *Adapted from Burrell et al. (2016)*

Inclusions of TDP43 protein are the main component of the ubiquitinated depositions found in FTD patients (Neumann et al., 2006). This same study showed that TDP43 inclusions are also found neuropathologically in ALS. TDP43 pathology varies in morphology and distribution in four different identified subtypes (**Figure 11**): type A is characterized by TDP43 immunoreactive neuronal cytoplasmic inclusions (NCI) in long neuritic profiles in superficial cortical layers; type B present TDP43-NCIs throughout the cortical layers; type C present inclusions in long dystrophic neurites; and in the rarer type D, inclusions are nuclear (Burrell et al., 2016; Chen-Plotkin et al., 2010; Tan et al., 2013). TDP43 inclusions in ALS have a different distribution pattern and are thus recognized as a fifth type of pathology (Burrell et al., 2016). These histopathological subtypes account for clinical presentations: about 50% of cases of nvPPA present type A pathology; 90% of cases with svPPA present type C pathology, and type D is found in patients with *VCP* mutations, whereas type B accounts for about 70% of FTD-motor neuron disease (MND) and 25% of bvFTD (Bang et al., 2015; Burrell et al., 2016).

Cases presenting *C9orf72* mutations displayed large NCIs in the granular layer of the cerebellar cortex and neuronal-intranuclear inclusions (NII) in pyramidal layers of the hippocampus that stain for ubiquitin and p62, but not for TDP43 (Mackenzie et al., 2014).



**Figure 12** Schematic representation of TDP43, p62 and DPR inclusions type

Prevalence and anatomical localization in FTD patient brains *Adapted from Schipper et al. (2015)*

Lesion load of p62 in *C9orf72* patients is much more pronounced than TDP43 pathology (Al-Sarraj et al., 2011; Schipper et al., 2015). Additionally, *C9orf72* cases present large nuclear RNA foci containing dipeptide repeats proteins (**Figure 12**).

#### 1.4. Genetics

Genetically, FTD is a complex disease. A large familial history, with an autosomal dominant pattern of inheritance present in 20 to 50% of cases, has branded FTD as a disease with a high genetic component (Rohrer et al., 2013, 2009). Mutations in *C9orf72*, *MAPT* and *PGRN* genes account for about 60% of all cases of inherited FTD, although a number of other genes are rare causes (**Table 2**) (Bang et al., 2015).

It was in 1998 that the first mutation in microtubule associated protein tau (*MAPT*) gene on chromosome 17 was discovered as causative of FTD (Hutton et al., 1998). Since then, more than 50 mutations in *MAPT* have been reported, accounting for 10% of all familial cases of FTD (Rademakers et al., 2012). In 2006, mutations in the progranulin (*PGRN*) gene were found to cause bvFTD in about 15% of familial cases (Pickering-Brown, 2007) and more recently in 2011, *C9orf72* gene mutations account for about 25% of cases of familial FTD (DeJesus-Hernandez et al., 2011; Renton et al., 2011; van der Zee et al., 2013) and 6-7% of sporadic cases, making it the most common genetic cause (Hardy and Rogaeva, 2014).

FTD-Tau has a remarkably variable clinical presentation. Patients can present either a pure dementia or present parkinsonian or amyotrophic features (Hardy and Orr, 2006). Brain atrophy is symmetric in the frontal lobe and also takes place in the anterior cingulate cortex, the insular, anterior and medial temporal lobes. Mutations occur in the coding sequence and mainly in exon-10 of *MAPT* gene, causing microtubule stabilization, disrupting axonal transport eventually leading to tau deposition and neurodegeneration (Bang et al., 2015). Abnormal accumulation of neuronal intermediate filaments in neurons could be a consequence of protein synthesis deregulation, failure of axonal transport, abnormal phosphorylation and proteolysis (Hochgräfe et al., 2013).



**Table 2** Identified genes causing familial FTD

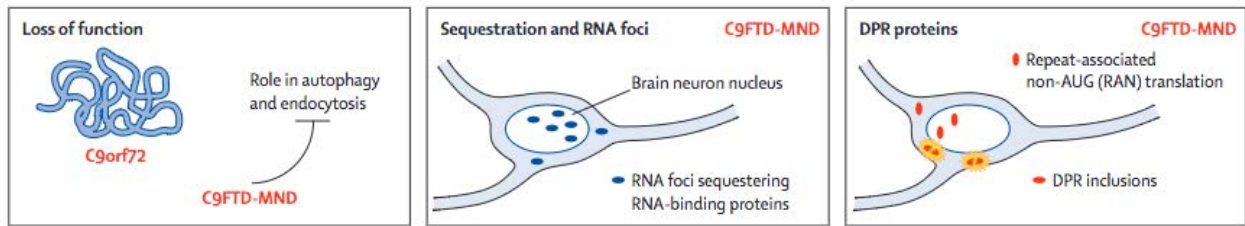
<i>Genes</i>	<i>Protein</i>	<i>Function</i>	<i>Common clinical presentations</i>	<i>Pathology</i>	<i>Frequency</i>	<i>References</i>
<b><i>C9orf72</i></b>	C9orf72	Unknown, vesicular transport, inflammation	bvFTD, PNFA, SD, ALS, c9FTD/ALS, parkinsonism, late onset psychosis	FTD-TDP (Type-B)	8-25%	Renton et al. 2011 DeJesus Hernandez et al. 2011
<b><i>PGRN</i></b>	Progranulin	Inflammation, cellular repair	bvFTD, PNFA, (SD), parkinsonism	FTD-TDP (Type-A)	15%	Cruts et al. 2006 Baker et al. 2006
<b><i>MAPT</i></b>	Microtubule associated protein Tau	Microtubule assembly and stabilization	bvFTD, PNFA, SD, parkinsonism	FTD-Tau (PiD, CBD, PSP, AGD, NFT-dementia, MSTD, MT-GGI)	5-20%	Hulton, 1998
<b><i>TARDBP</i></b>	TDP43	RNA metabolism	(bvFTD), ALS, FTD/ALS	FTD-TDP	2%	Kabashi et al. 2008
<b><i>VCP</i></b>	Valosin-contain protein	AAA-ATPase	bvFTD	FTD-TDP (Type-D)	5%	Watts et al. 2004
<b><i>FUS/TLS</i></b>	Fus	RNA metabolism	ALS, (bvFTD) FTD/ALS		<1%	Vance et al. 2009
<b><i>CHMP2B</i></b>	Charged multivesicular body protein 2B	Multi-vesicular body formation	bvFTD, FTD/ALS, (parkinsonism)	FTD-UPS	<1%	Skibinski et al. 2005
<b><i>SQSTM1</i></b>	p62	Autophagic degradation of ubiquitinated proteins	FTD, ALS, FTD/ALS	FTD-TDP	3%	Rubino et al. 2012 Le Ber et al. 2013 Fecto et al. 2011
<b><i>UBQLN2</i></b>	Ubiquilin	Ubiquitylation and proteasomal degradation	ALS, FTD/ALS	Skein-like inclusions UBQLN2, ubiquitin, p62, TDP43, FUS, OPTN		Deng et al. 2011
<b><i>OPTN</i></b>	Optineurin	Autophagy receptor	ALS, FTD/ALS	TDP (Type B) OPTN in motor neurons	<1%	Maruyama et al. 2010
<b><i>TBK1</i></b>	Tank binding kinase 1	NFkB activation, inflammation	FTD	?		Borghero et al. 2016

Mutations in progranulin are broadly distributed throughout the gene, being most of them loss-of-function mutations that segregate in an autosomal dominant mode of inheritance. Patients present nuclear and cytoplasmic TDP43 inclusions with asymmetrical, anterior temporal, temporo-parietal, frontal, anterior cingulate and insular cortex atrophy (Bang et al., 2015; Hardy and Rogaeva, 2014). Progranulin is a secreted protein involved in cell-cycle regulation, axonal growth and modulation of inflammation. It binds to the tumor necrosis factor (TNF) receptor antagonizing TNF $\alpha$ -induced neuroinflammation (Bang et al., 2015; Cenik et al., 2012; Jian et al., 2013; Tang et al., 2011). Furthermore, progranulin is capable of activating the endosomal-autophagosomal-lysosomal pathway and might have a role in clearing TDP43 aggregates (Hardy and Rogaeva, 2014).

Mutations in *TARDBP* and *FUS* genes have also been associated to FTD and ALS. These two RBPs have parallel roles in the cell, and they may act in common pathways, particularly in RNA regulation (Lagier-Tourenne et al., 2010). A minimal proportion of cases present mutations in *VCP*, *CHM2B*, *SQSTM1* (*p62*), *OPTN*, *UBQLN2* and *TBK1* genes, altering autophagy and/or proteasomal processes and neuroinflammation (Borghero et al., 2016 ; Hardy and Rogaeva, 2014).

In 2011, two independent teams identified the intronic GGGGCC expansion in chromosome 9 open reading frame 72 (*C9orf72*) as the most common cause of both familial and sporadic FTD, also being the most common cause of ALS (DeJesus-Hernandez et al., 2011; Gijssels et al., 2012; Renton et al., 2011; van der Zee et al., 2013). Patients present symmetrical, orbitofrontal and dorsolateral frontal atrophy followed by temporal lobes, parietal and occipital lobes, cerebellum and posterior thalamus (Bang et al., 2015). Postmortem examination shows TDP-43 positive inclusions widely distributed through the brain, p62 positive- TDP43 negative inclusions in the cerebellar granular layer; hippocampal pyramidal neurons and other sites and presence of dipeptide repeat pathology mainly in the cerebellum (polyGA being the most common in the brain) (Mackenzie et al., 2015). The *C9orf72* protein has been predicted to be a differentially expressed in normal and neoplastic cells (DENN) protein acting as a guanosine exchange factor (GEF), localizes mainly to the nucleus and can regulate membrane trafficking (Burrell et al., 2016; Farg et al., 2014; Levine et al., 2013). The disease mechanism caused by the expansion has not been fully elucidated. Several groups have observed a reduced expression of *C9orf72* transcripts, suggesting a loss-of-function mechanism. Furthermore, RNA-foci formation has been identified in patients' brains and spinal cord suggesting a possible toxic RNA gain of function disease mechanism.

And finally, translation of repeats into dipeptide-repeat proteins that aggregate could also have a toxic effect (**Figure 13**).



**Figure 13** Possible diseases mechanisms of the C9orf72 repeat expansion.

(a) Protein loss-of-function impairing autophagy and endocytosis. (b) Sequestration of RNA-binding proteins depleting the cell of these proteins. (c) DPR proteins forming intracellular inclusions disturbing neuronal function. *Adapted from Burrell et al. (2016)*

## 2. Features of amyotrophic lateral sclerosis

### 2.1. Epidemiology, clinical features and genetics

Amyotrophic lateral sclerosis is a motor neuron degenerative disease. It was Jean-Martin Charcot, the famous French neurologist, who firstly described the relation between the motor clinical symptoms to the underlying neurological alteration. In 1874, he introduced for the first time the term “amyotrophic lateral sclerosis”, but the disease was commonly known as “Charcot’s disease”.

ALS affects 2 in 100,000 people in Europe, and more than 5,600 people are diagnosed with it in the United States (Kiernan et al., 2011). Worldwide, rates of ALS are mostly unknown, but there seemed to be areas with a higher prevalence. Great interest emerged in the 1950s in the Pacific Rim after a huge epidemic outbreak occurred in the Chamorro people on Guam. During this outbreak, 400 per 100,000 Chamorro suffered from the neurological syndrome of amyotrophic lateral sclerosis/parkinsonism-dementia complex (ALS-PDC)(Lee, 2011). With this high incidence, the hypothesis of an environmental factor involved in the development of ALS-PDC was then proposed. Investigators found that the incidence may be linked to the unwary consumptions of neurotoxin  $\beta$ -methylamino-L-alanine (BMAA) produced by cyanobacteria living in the seeds of cycad trees. Chamorro people feast on flying fox bats that nourish on these seeds (Mitchell and Borasio, 2007). BMMA is an excito-toxin that will cause persistent excitation of neurons leading to exhaustion and death (Cox and Sacks, 2002).

ALS is classified as a rare disease, but it is the most common motor neuron disease, affecting people from all ethnics and races. Male seem to be more affected than females (ration 1,6 to 1) (Mitchell and Borasio, 2007). The course of the disorder is progressive, with 50% of patients dying within 3 years after onset (Robberecht and Philips, 2013). Remaining cases present 20 or more years of survival (Turner et al., 2013). Up to 90% of cases lack a genetic component and are known as “sporadic ALS”, whereas 5-10% of cases are caused by mutations in specific genes (Chen et al., 2013). In familial ALS, 15-20% or patients have mutations in the Cu/Zn *SOD1* (Chen et al., 2013). Mutations in the *TARDBP* gene and *FUS* represent 5% of familial ALS patients (Parakh and Atkin, 2016) and mutations in *C9ORF72* gene represent the most common cause of both familial and sporadic ALS cases, with 37% and 7% respectively (DeJesus-Hernandez et al., 2011; Renton et al., 2011). Although more rare, mutations in *ANG* coding for angiogenin and *OPTN* coding for optineurin also cause typical ALS (**Table 3**). Emerging evidence starts pointing towards a more complex genetic cause with the presence of a phenotypical variability that denotes vulnerability to ALS, rather than a sole major mutation driving pathology (Turner et al., 2013).

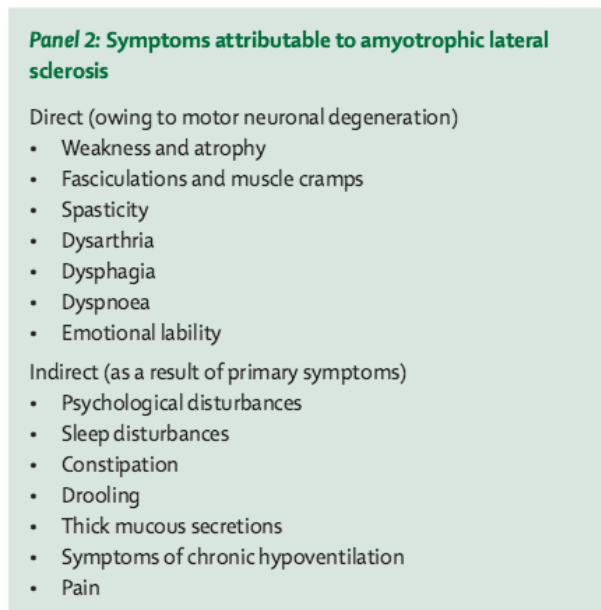
**Table 3** Identified genes causing familial ALS

<i>Genes</i>	<i>Protein</i>	<i>Function</i>	<i>Common clinical presentations</i>	<i>Pathology</i>	<i>Frequenc y</i>	<i>References</i>
<b><i>C9orf72</i></b>	C9orf72	Unknown	bvFTD, PNFA, SD, ALS, c9FTD/ALS, parkinsonism, late onset psychosis	FTD-TDP (Type-B)	8-25%	Renton et al. 2011 DeJesus Hernandez et al. 2011
<b><i>SOD1</i></b>	Super oxide dismutase 1	Apoptosis, binding cooper and zinc ions	ALS	SOD1	12-23%	Rosen et al. 1994
<b><i>TARDBP</i></b>	TDP43	RNA metabolism	(bvFTD), ALS, FTD/ALS	FTD-TDP	2%	Kabashi et al. 2008
<b><i>FUS/TLS</i></b>	Fus	RNA metabolism	ALS, (bvFTD) FTD/ALS		<1%	Vance et al. 2009
<b><i>ANG</i></b>	Angiogenin	RNA metabolism	ALS	-	<1%	
<b><i>CHMP2B</i></b>	Charged multivesicular body protein 2B	Multi-vesicular body formation	bvFTD, FTD/ALS, (parkinsonism)	FTD-UPS	<1%	Skibinski et al. 2005
<b><i>SQSTM1</i></b>	p62	Autophagic degradation of ubiquitinated proteins	FTD, ALS, FTD/ALS	FTD-TDP	3%	Rubino et al. 2012 Le Ber et al. 2013 Fecto et al. 2011
<b><i>UBQLN2</i></b>	Ubiquilin-2	Ubiquitylation and proteasomal degradation	ALS, FTD/ALS	Skein-like inclusions UBQLN2, ubiquitin, p62, TDP43, FUS, OPTN		Deng et al. 2011
<b><i>OPTN</i></b>	Optineurin	Autophagy receptor NFkB activation	ALS, FTD/ALS	TDP (Type B) OPTN in motor neurons	<1%	Maruyama et al. 2010

## 2.2. Diagnosis

This fatal neurodegenerative disease is characterized by a degeneration of upper and lower motor neurons that give rise to progressive spasticity, muscle wasting, and weakness (Kiernan et al., 2011; Mitchell and Borasio, 2007). Increasingly, ALS is being considered as a complex multisystem disease as at least 50% of patients undergo degeneration in other areas of the brain than the motor cortex (Kiernan et al., 2011; Parakh and Atkin, 2016; Turner et al., 2013). It is now widely accepted that ALS depends on multiple factors, including the initial

clinical presentation itself (phenotype) but also the rate of disease progression, the early presence of respiratory failure and the nutritional status of patients (Kiernan et al., 2011). Clinical presentations are varied and they are accounted according to the attained regions (**Figure 14**) Early manifestations can arise at a bulbar, cervical or lumbar level (Kiernan et al., 2011; Mitchell and Borasio, 2007). Bulbar-onset patients present dysarthria or slurring of speech and/or difficulty in swallowing or dysphagia. Facial weakness and poverty of palatal movement with fasciculation of



**Figure 14** ALS common symptoms

*From Mitchell and Borasio, (2007)*

the tongue can also be presented in bulbar palsy. Patient with cervical disease onset present difficulties in upper-body movements associated with shoulder abduction, like hair washing, and impairment in activities that require gripping. Lumbar onset is associated with signs and symptoms in the legs, such as tripping or difficulty with stair climbing. Determining specific phenotypes allows to identify prognosis and survival and to implement a better patient care. Some phenotypes have been related to specific mutations. For example, patients with the C9orf72 HRE present decreased survival rates compared to other forms of the disease, whereas mutations of *SOD1* A4V will cause a rapidly progressive primarily lower motor neuron syndrome, and *FUS* mutations will cause more commonly a juvenile onset (Turner et al., 2013). To date, diagnosis is made by clinicians, as no specific biomarker has been identified. Muscle biopsies, nerve conduction studies and electromyogram are valuable tools for excluding unusual myopathies and confirming the ALS diagnosis (Kiernan et al., 2011). Motor nerve conduction declines as disease progresses, but sensory nerve conduction remains normal in ALS patients. The presence of fibrillation potentials, positive sharp waves and chronic neurogenic changes in the electromyogram indicate lower motor neuron loss and the presence of subclinical involvement (**Figure 15**) (Kiernan et al., 2011). MRI does not contribute to ALS diagnosis. Cerebral atrophy can be detected, if present. The use of magnetic resonance allowed for the identification of neuronal loss, particularly in the primary motor cortex. Functional studies with PET imaging have allowed to identify extra-motor changes, particularly frontal deficits leading to a FTD-ALS diagnosis (Kew et al., 1993).

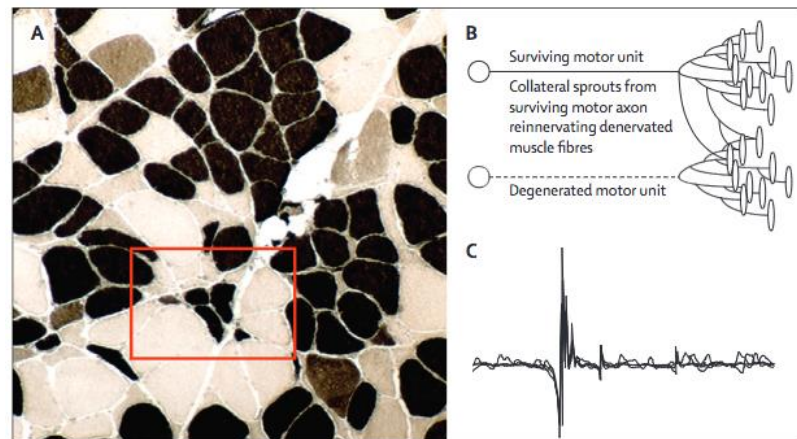
Receptor ligand PET results have shown a reduced inhibitory GABAergic output in the cortex of patients, supporting the hyperexcitability hypothesis in ALS (Kiernan et al., 2011).

Age of onset, low forced vital capacity, time from first symptom presentation and bulbar-onset are adverse prognostic indicators (Mitchell and

Borasio, 2007). Environmental risk factors for ALS have been suggested, such as exposure to childhood infections, extensive sport or physical exertion. Weight and food intake are also carefully monitored in ALS patients, as there is a strong metabolic component. Patients are generally lean and lose body mass, muscle mass, and fat as the disease progresses, which is linked to the observation of hypermetabolic state in 50-60% of patients (Kiernan et al., 2011; Turner et al., 2013). Metabolic changes, such as insulin resistance, fluctuations in weight and cholesterol, have a higher incidence in ALS patients, but if these changes are a secondary effect of disease development or progression, or causative has yet to be elucidated (Ahmed et al., 2016). Following these observations, the hypothesis whether patients with high premorbid body mass index (BMI) develop ALS with cognitive deficits while those with a low BMI develop pure ALS has been raised (Bouteloup et al., 2009). Nutritional advice is programmed on the multidisciplinary care model proposed nowadays (Kiernan et al., 2011; Mitchell and Borasio, 2007).

### 2.3. Neuropathology

ALS pathology is characterized by the presence of intracellular, insoluble inclusions composed of misfolded proteins detected in the brain and spinal cord of affected patients. Familial forms of ALS present intracellular protein inclusions of misfolded proteins encoded by the mutated genes within motor neurons. SOD1, TDP43 and FUS proteins misfold and

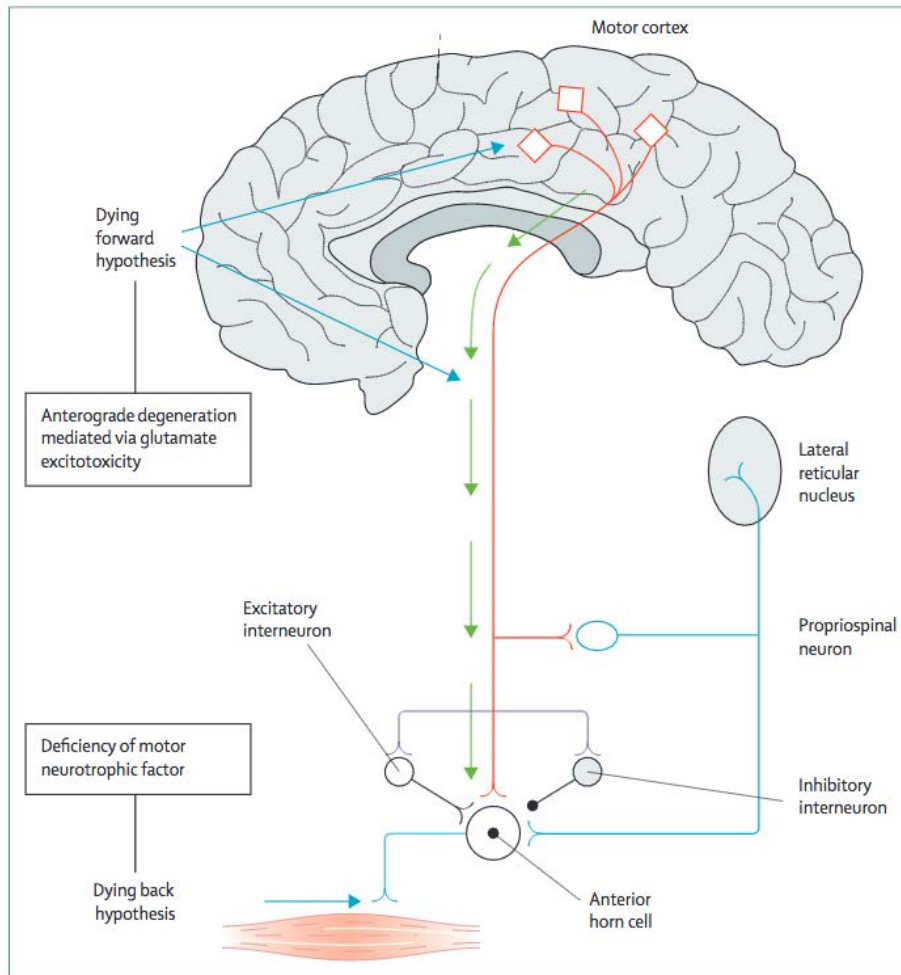


**Figure 15** Tools for ALS differential diagnosis

(a) muscle biopsy showing mixed-types fibers -stained with ATPase pH 9.4- (b) motor unit degeneration and reinnervation model (c) EMG unstable traces of motor unit in ALS *Adapted from Kiernan et al. (2011)*

aggregate in the affected tissues (Parakh and Atkin, 2016). Besides TDP43 pathology and p62 aggregates, C9orf72 mutations carriers present abnormal dipeptide repeat proteins inclusions resulting from unconventional repeat-associated translation (Ash et al., 2013; Mori et al., 2013b). Possible pathophysiological mechanisms leading to ALS pathology describe implications of glutamate-induced excitotoxicity, mitochondria abnormalities, dysfunction of the sodium potassium ion pump, autophagy and disrupted axonal transport system, plus involvement of non-neuronal cells (astrocytes and microglia) (Boillée et al., 2006a; Kiernan et al., 2011). Furthermore, primacy of lower motor neuron degeneration over upper motor neurons remains an issue of debate, as many initial changes occur in the periphery but upper motor neuron degeneration seems dominant raising the question to whether the disease mechanism occurs in a “dying-forward” or “dying-back” manner (**Figure 16**) (Turner et al., 2013).





**Figure 16** The “dying-forward” and “dying-back” motor neuron hypothesis.

Toxicity will be lead by glutamate-induced excitotoxicity (green arrows) as upper motor neurons mediate anterograde degenerate of lower motor neurons. Insufficient release of neurotrophic factors, secretion of neurotoxic mediators could also lead to degeneration. *Adapted from Kiernan et al. (2011)*

### 3. How have a dementia and a motor neuron disease been linked together?

In 1892, Dr. Pierre Marie, at the time student of Pr. Charcot, described for the first time the observation that dementia was often accompanied by motor neuron disease (MND) (Marie, 1892). But it was only until the early 1990s that awareness of the links between these two pathologies was rekindled. Hudson's observations in 1991 showing an important number of patients developed both ALS and dementia in Guam helped putting forward the idea of a possible mechanistic relation between these two pathologies (Hudson, 1991). During the next years, several cases of MND with dementia associated with a degeneration of the frontal lobe were described. The report of Lomen-Hoerth et al. in 2002, in which 15% of FTD patients suffer from ALS, helped anchoring further the idea that FTD and ALS are two opposite ends of the same disease spectrum. Since that time, more genetic markers, neurological and clinical signs have emerged confirming the overlap between these two pathologies (**Figure 17**).



**Figure 17** Schematic representation of the FTD and ALS continuum

FTD and ALS represent two opposite ends of a same disease spectrum. Common genes display different clinical presentations. Genes implicated in autophagy and proteasome are in red. Genes implicated in RNA metabolism are in blue.

Nevertheless, controversy surrounding the idea of a link persisted for several years. The fact that *SOD1* mutations caused ALS with no FTD associated symptoms created reticence in the ALS scientific community to accept both syndromes as one with different clinical presentations. An increasing number of studies showed that relatives of patients with ALS had a higher risk of developing dementia than the standard population, and that patients with MND develop cognitive symptoms (Hokezu et al., 1990; Lomen-Hoerth et al., 2002). The discovery that ALS patients develop spongiform degeneration in the frontal and

precentral gyri (cortical layers II and III), diffuse subcortical gliosis and cortical inclusions of TDP43 protein boosted acceptance of the FTD-ALS continuum. Furthermore, an association between both diseases in sporadic cases also seem to be common (Ng et al., 2014). Multidisciplinary evaluation of patients has allowed identifying presence of both syndromes in the same families, marking a significant shift in the field.

Nowadays, research groups study both pathologies in parallel, particularly since the discovery of the GGGGCC HRE in the gene *C9orf72*. Several families have been described in which affected individuals clinically manifest FTD alone, ALS alone or both.

#### **4. The *C9orf72* repeat expansion, tying up FTD and ALS**

The *C9orf72* HRE has a frequency of 25% in familial FTD and 40% in familial ALS FTD, with around 6% in sporadic FTD cases and 9% in sporadic ALS cases (Ng et al., 2014; Renton et al., 2011; van der Zee et al., 2013). Mutation frequency varies within different populations, with the highest prevalence in the Caucasian populations and in Northern Europe populations in general (Majounie et al., 2012; Ng et al., 2014). This observation raised the hypothesis of a single Scandinavian founder and the fact that geographically separated and unrelated families shared a common haplotype supported further this idea. However, no north-south axis has been fully identified and additional findings support rather the hypothesis of multiple origins for the expansion, particularly since cases of patients carrying the mutation with no familial relation to ALS or FTD have been reported (Xi et al., 2015a). This second hypothesis proposes that a specific genomic context will render the repeat less stable making it prone to expansion. Observation of pre-mutation carriers (asymptomatic carriers with over >30 repeats) having offspring presenting large expanded *C9orf72* repeats (~1700 repeats) supports further this idea (Xi et al., 2015a, 2015b). However, in a second independent study of an Australian ALS-trio, no evidence of intergenerational repeat amplification was identified (Pamphlett et al., 2012). Further research will yet be necessary to determine the origin of the *C9orf72* expansion. While control populations show a range of 2-30 repeats, ALS and FTD patients harbor an estimate of 700 to 1600 repeat-units (DeJesus-Hernandez et al., 2011). The effect of 30 to 300 repeats remains mysterious, as some cases may develop the disease whereas others do not, and no association with the disease has been established (van der Zee et al., 2013). A risk effect for these intermediates repeats has been considered, as a correlation between repeat size with onset age of disease has been made (Gijssels et al.,

2015). In this study, short expansion carriers showed a later onset of disease with significant lower levels of methylation in the 5' flanking CpG island.

Age of onset and disease duration are highly variable in c9FTD/ALS, ranging from 27 to 83 years for onset and 3 months to over 15 years of disease duration (Majounie et al., 2012; Ng et al., 2014). Prognosis data suggest that C9orf72 expansion carriers have shorter survival than other FTD or ALS patients (Simon-Sanchez et al., 2012). Clinically, C9orf72 patients seem to present bulbar onset ALS, cognitive impairment with early onset and accelerated progression than patients without the expansion (Ling et al., 2013). Studies have found that patients with the C9orf72 mutation can present additional features to FTD-ALS pathology such as memory disorders, psychosis, akinetic-rigid syndrome and cerebellar signs (Ng et al., 2014; Rademakers et al., 2012). Symptoms of psychiatric diseases were commonly seen in C9orf72 cases, including borderline personality, antisocial and childishness behaviors and bipolar illness, with language impairments and parkinsonism (Gómez-Tortosa et al., 2016; Le Ber, 2015). Differences between ALS and FTD seem to vary according to affected cerebellar sub-regions, with ALS cases showing atrophy rather in the inferior cerebellum and vermis, which associates with motor dysfunction, while FTD cases present widespread atrophy mainly in the superior cerebellum (R. H. Tan et al., 2014)

#### **4.1. Transcripts and protein variation in C9orf72 carriers**

The particular localization of the C9orf72 repeat, upstream of the first exon or in the promoter region depending on transcript, seems to alter gene expression. Several reports have described a 50% loss of transcript levels, particularly in the frontal cortex and the cerebellum (Blitterswijk et al., 2015; DeJesus-Hernandez et al., 2011; Fratta et al., 2013; Gijssels et al., 2012; Renton et al., 2011; van der Zee et al., 2013). Moreover, higher levels of transcript variant 1 were associated with longer survival (Blitterswijk et al., 2015), suggesting a damaging effect in loss-of-function of C9orf72.

A somatic mosaicism has been observed among different tissues seen in patients. Furthermore, the intra-individual variation of repeats-number between tissues is higher than the variation within each tissue group (Nordin et al., 2015). Regardless of clinical phenotype, the expansion lengths were always larger in the frontal lobes and spinal cord than in the cerebellum but a clear correlation between their presence with clinical disease presentation has not been established yet (van Blitterswijk et al., 2013).

Genetic variations in other genes may drive the disease pathology toward a specific side of the clinical FTD/ALS continuum e.g. ATXN2 which increased risk in C9orf72 expansion carriers for ALS, or TMEM106 which has been identified as a risk factor for FTD-TDP (Lattante et al., 2015; Ng et al., 2014), raising the question of oligogenic inheritance that will act as disease modifiers events.

#### **4.2. Inclusions in C9orf72 patients**

TDP43 pathology in C9orf72 expansion cases is found in temporal and frontal cortices, in limbic (hippocampus and amygdala), brain stem (midbrain/*substantia nigra*) and subcortical structures (striatum and thalamus) and consist of compact and granular NCI, dystrophic neurites (DNs), glial cytoplasmic inclusions, and variable presence of NIIs (Murray et al., 2011). Most inclusions are type B TDP43 pathology in all cortical layers. In clinical ALS, inclusions were found in neurons and glia in the ventral gray matter of the spinal cord (Hsiung et al., 2012). The observed clinical phenotype seems to correlate with the degree of TDP43 pathology and degeneration in the affected brain regions.

C9orf72 expansion cases also show presence of ubiquitin positive, TDP43 negative inclusions in the cerebellar granule cell layer, hippocampal pyramidal neurons and neocortex. These ubiquitin positive inclusions stain for p62 and also for dipeptide-repeat proteins produced by RAN translation of sense and anti-sense transcripts of the abnormal expanded repeat. The most common forms of dipeptide proteins present in patient's brains are poly (GA) and poly (GP) proteins, with a lower presence of poly (GR) proteins. They are mostly located in the hippocampus, the cerebellum, the thalamus and the neocortex (Ash et al., 2013; Blitterswijk et al., 2015; Mackenzie et al., 2015; Mori et al., 2013b).

Although the FTD-ALS continuum starts to be elucidated from a clinical perspective, as now we are able to identify diverse clinical phenotypes, molecular mechanisms that account for this heterogeneity remain unclear. Diverse forms of FTD and the fact that some ALS cases present cognitive deficits have allowed for a better patient care. Identification of genes linking these two pathologies has given great insight into the possible converging pathways that lead to each pathology, with possible inter-dependent relationships in the manifestation of diverse clinical phenotypes. Furthermore, the fact that ALS linked to SOD1 mutations seem to have a different disease pathway, has open the door to new considerations of disease pathology. Little by little, we have begun to dissect underlying mechanisms of neurobehavioral deficits specific to each disorder to better understand differences between FTD, ALS and ALS/FTD. In the next chapter, I will present current knowledge on underlying molecular mechanisms.

## IV. Underlying physiopathological mechanisms in FTD and ALS

We have been trying to understand cellular and molecular mechanisms that drive pathogenesis in ALS and separately, in FTD for over a century. Mounting evidence has pointed towards a phenotypical continuum between these two disorders, each representing one of the extremes of the same disease spectrum. Pathologically, the majority of both ALS and FTD cases are characterized by cytoplasmic inclusions of TDP43 protein in the brain and spinal cord, with the co-occurrence of nuclear TDP43 depletion (Jovičić et al., 2016) (similar observations have been made for FUS pathology, but will not be discussed here). The discovery of C9orf72 mutations has further reinforced this overlap between ALS and FTD, as they seem to share common disease mechanisms. Mutated genes that cause FTD and ALS can be separated in two major functional groups: those associated with RNA metabolism (*TDP43*, *FUS*) and those affecting protein degradation (*UBQLN2*, *SQSTM1*, *OPTN*, *VCP*). Pathogenesis could be caused by alterations in either or both groups. Convergence of both seems the most plausible path, as patients present disturbances in multiple cellular pathways, but which events will stir disease onset remains unknown. In this chapter, I will explore current knowledge in RNA and protein homeostasis alteration in the context of FTD, ALS and ALS/FTD with the example of TDP43 and C9orf72 HRE.

### 1. Implications of TDP43 in FTD and ALS and its role as an mRNA binding protein

Even though TDP43 abnormal cellular distribution and post-translational modifications are key markers of FTD and ALS, the underlying mechanisms and the role played by this aggregation are still unknown. Since Neumann et al. showed in 2006 that TDP43 is the main component of ubiquitin positive aggregates in neurons, several teams have joined efforts to elucidate why this aggregation occurs and how does it affect cell survival. The identification of more than 50 mutations in the *TARDBP* gene associated with FTD and ALS confirmed the direct link between TDP43 protein alterations and occurrence of disease, but whether and to what extent TDP43 aggregation plays a role in disease origin and progression is still debated. Some think that TDP43 pathology is the most important cellular pathway that leads to neuronal impairment or death while others propose that it acts mostly as a disease modifier rather than a driver of disease course (Buratti, 2015; Chen-Plotkin et al., 2010). As TDP43 pathology is present in over 50% of FTD cases and 97% ALS cases (Jovičić et al., 2016; Mackenzie et al., 2010; Neumann et al., 2006; Parakh and Atkin, 2016), it is now crucial to understand what are the functional consequences of mutations. Additionally, it is important to

identify what triggers TDP43 aggregation and if targeting this pathway could lead to the development of new disease-modifying therapies. In this chapter, I will present current knowledge of TDP43 pathology, and of the basic properties and medical relevance of TDP43.

### 1.1. *TARDBP* gene and TDP43 protein

TDP43 is a 414 amino acid protein of 43kDa encoded by the *TARDBP* gene on chromosome 1 and is made up by 6 exons, which may be variably spliced to produce multiple isoforms. Even though bioinformatics studies predict the existence of at least eleven isoforms, only three isoforms have been detected in brain and spinal cord tissue, but their functional significance is still not known (Buratti and Baralle, 2008; Strong et al., 2007).

The gene is located in a highly conserved gene-rich region among species. This high degree of conservation hints towards a fundamental role played by the protein (Buratti and Baralle, 2008). Like many other similar proteins that belong to the family of hnRNP proteins, TDP43 plays a role in RNA metabolisms (Buratti, 2015; Chen-Plotkin et al., 2010). TDP43 can bind both DNA and RNA and act as a transcriptional repressor, be involved in splicing of several genes and play a role in RNA maturation, a process that involves shuttling of proteins between the nucleus and the cytoplasm. These processes are due to TDP43 ability to bind RNA in a sequence specific manner through two RNA binding domains (RRM1 and RRM2) (**Figure 19**). Binding specificities have been shown to regulate almost all the currently described protein functions: TDP43 preferentially binds GU-rich motifs in RNA and TG-repeated motifs in DNA, increasing binding affinity with the increase of repeated motifs number. Specific mutations in RRM1 are sufficient to abolish RNA-binding and splicing regulatory activity (Buratti and Baralle, 2001). The function of RRM2 remains elusive and does not appear to have a role in RNA-binding as its affinity for RNA is two-fold lower than that of RRM1, but it has been proposed to play a role in chromatin organization (Lagier-Tourenne et al., 2010). A possible role of RRM2 in TDP43 dimerization has also been suggested, as TDP43 forms dimers under physiological conditions, and this region is involved in protein-protein interaction and is capable of co-crystallizing with TG-rich DNA sequences forming highly stable dimeric assemblies (Kuo et al., 2009; Shiina et al., 2010).

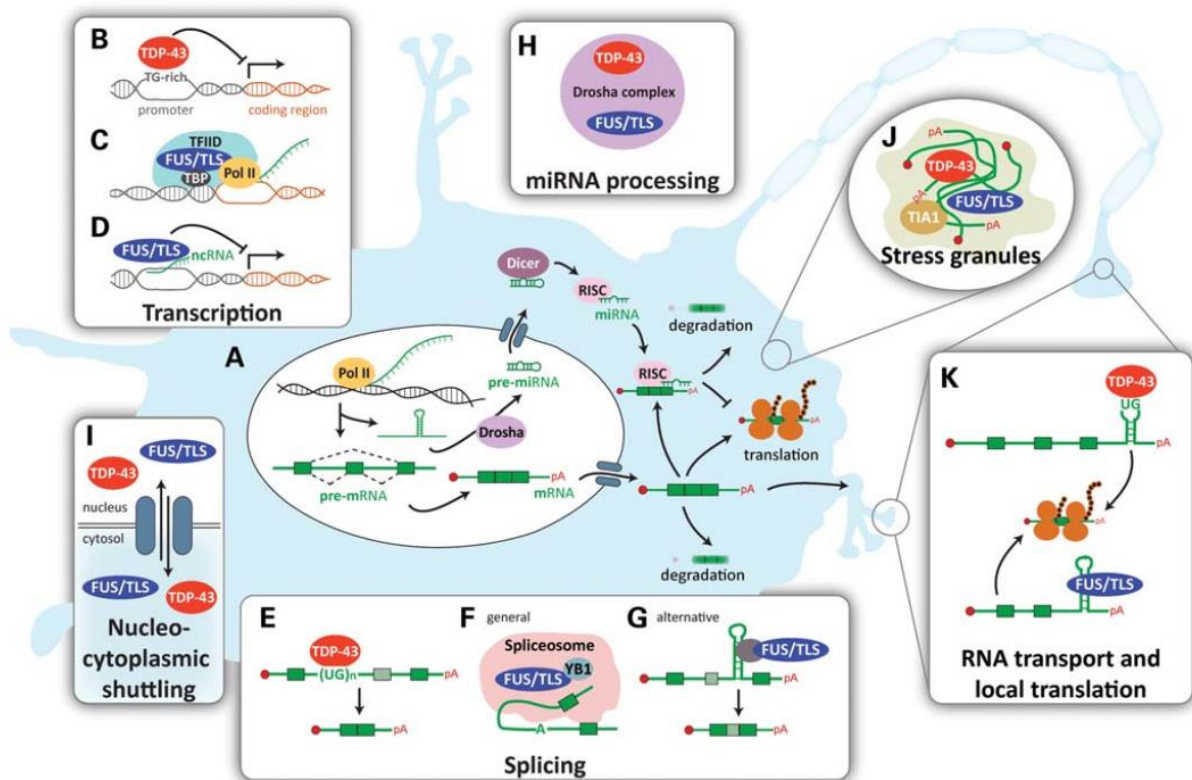
TDP43 possesses an N-terminal nuclear localization sequence and carboxy-terminal glycine-rich domain important for its role in gene splicing and protein-protein interaction. Indeed, contact with other members of the hnRNP protein family, such as hnRNP A1, A2/B1, and A3 has been ascribed to this region (Buratti and Baralle, 2008). Much less is known about



the N-terminal region of TDP43. It seems to be conserved more loosely than the RRM regions but it presents a NLS at aa position 82-98 and a NES at aa 239-25, extremely important for subcellular transport (Wang et al., 2008).

TDP43 is widely expressed in tissues including heart, liver and brain and has a nuclear localization, being normally localized to euchromatic regions of the nucleoplasm, including perichromatin fibrils and nuclear speckles where transcription and co-transcriptional splicing occur (Ayala et al., 2011; Lee et al., 2011; Neumann et al., 2006). Mislocalized TDP43 to the cytoplasm has been observed in stress conditions and in disease, however, a low degree of cytoplasmic TDP43 may be observed in normal cells. This is not surprising since the protein is synthesized in the cytoplasm and shuttles between the nucleus and cytoplasm (Buratti and Baralle, 2008). Like many other RBPs, TDP43 controls its own expression through a negative feedback loop requiring its binding activity through the specific association between the 3'UTR sequences and to the C-terminal region of the transcript (Ayala et al., 2011; Lee et al., 2011). Thus, overexpression of TDP43 leads to a decrease in endogenous TDP43 in cultured cells and transgenic mice.

As TDP43 has been shown to play an important role in several biological pathways, the exact cellular functions and how they can lead to pathology are still being characterized (**Figure 18**).



**Figure 18** Physiological roles of TDP43 (and FUS)

Roles in (A) RNA processing, (B-D) transcription regulation and (E-G) splicing; with roles in (H) miRNA processing, (I) nucleocytoplasmic shuttling regulation and (J) stress granules assemblage. Finally showing a role in (K) mRNA shuttling to dendrites for local translation *Adapted from Lagier-Tourenne et al (2010)*

Firstly identified as a transcriptional repressor of HIV1 gene expression, nowadays TDP43 is known to be implicated in multiple biological pathways (**Figure 18**). Roles such as regulating gene expression through transcription; selected splicing of several genes including the most well-known human cystic fibrosis receptor (*CFTR*), apolipoprotein A2 (*APOA3*) and survival motor neuron (*SMN*); and implications in mRNA stability, as happens with the human low molecular weight neurofilament (hNFL) transcript, the cyclin-dependent kinase 6 (CDK6) among others have been described (Chen-Plotkin et al., 2010; Lee et al., 2011; Sephton et al., 2011; Strong et al., 2007). Of notice, splicing alterations and mRNA-editing errors have been reported in sporadic ALS patients, but a role of TDP43 in these modifications has not been explored (Lagier-Tourenne et al., 2010). Furthermore, the localization of TDP43 in RNA granules within neuronal process also suggests involvement in RNA trafficking (Hörnberg and Holt, 2013). Different RBPs allow for packaging and transport of inactive mRNAs towards distal sites for local translation and towards post-

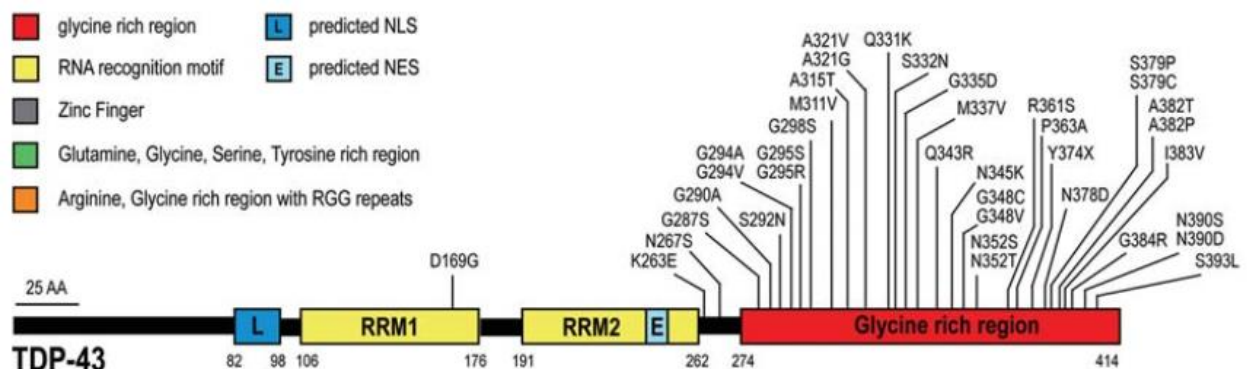
synaptic regions. In the same manner, dormant RNAs are assembled by RBPs into polysomes for local protein synthesis following neural activation (Govindarajan et al., 2006; Wang et al., 2008) TDP43 is found in RNA transporting granules translocating towards dendritic spines upon different stimuli (Alami et al., 2014). Increasing evidence suggest that TDP43 is an integral component of stress granules (Chen-Plotkin et al., 2010; Lagier-Tourenne et al., 2010). Induced cellular stress causes the redistribution of TDP43 from the nucleus into the cytoplasm, and stressors like sodium arsenate cause TDP43 cytoplasmic aggregation or relocalization to stress granules. Stress granules are cytoplasmic sites of stalled mRNA pre-initiation complexes. As arsenate reacts with oxygen, it induces oxidative stress that activates heme-regulated inhibitors, which expression will cause stress granule formation (McDonald et al., 2011; Meyerowitz et al., 2011). TDP43 is neither essential for formation of stress granules nor neuroprotective under stress conditions, although it contributes to their formation (Aulas et al., 2012). It is suggested that TDP43 will mediate stabilization and transport of hNFL mRNA toward an injured site for local translation of hNFL protein required for axonal repair (Lagier-Tourenne et al., 2010; Strong et al., 2007)

Finally, it has been shown that TDP43 interacts with FUS (Ling et al., 2010). This interaction is less than 1% when both proteins are normal, but mutations causing ALS will highly enhance this association. FUS bears strong similarities with TDP43; both can bind RNA and DNA, having a function in regulation of RNA splicing and gene expression, and thus raising the question of abnormal RNA processing and its relation to disease course. Aside from its role in mRNA pathways, TDP43 also regulates microRNA (miRNA) biogenesis binding directly the Drosha complex involved in miRNA maturation; and this has also been observed for FUS. In addition, TDP43 can also associates with proteins of the Dicer complex and having a possible role in cytoplasmic cleavage of miRNA (Lagier-Tourenne et al., 2010; Lee et al., 2011; Ling et al., 2010). Taken together, over 300 factors have been identified to interact with TDP43, composing the protein's interaction network and thus explaining the potential diversity of TDP43's cellular functions (**Figure 18**) (Freibaum et al., 2010; Sephton et al., 2011; Tollervey et al., 2011).

## 1.2. Pathological TDP43

### 1.2.1. *TARDBP* mutations

TDP43 aggregates are observed in cases with no mutations of the *TARDBP* gene. Nevertheless, an intensive search for mutations in the gene took place to establish a clear molecular link with disease. In 2008 several teams identified ALS and FTD causing mutations in the *TARDBP* gene (Borroni et al., 2009; Cairns et al., 2007). Mutations in *TARDBP* gene are rare and difficult to identify; they stand for approximately 2-3% of sporadic and familial forms of FTD and ALS. To date, 52 mutations have been identified, presenting an autosomal dominant pattern of inheritance and being missense mutations. The vast majority of these mutations reside in the glycine rich domain, while very few mutations happen in other regions (Figure 19) (Buratti, 2015). This prevalence of mutation in the glycine-rich domain may be due to the fact that mutations in less well conserved regions may affect TDP43 function and thus alter development, making them lethal embryonically.



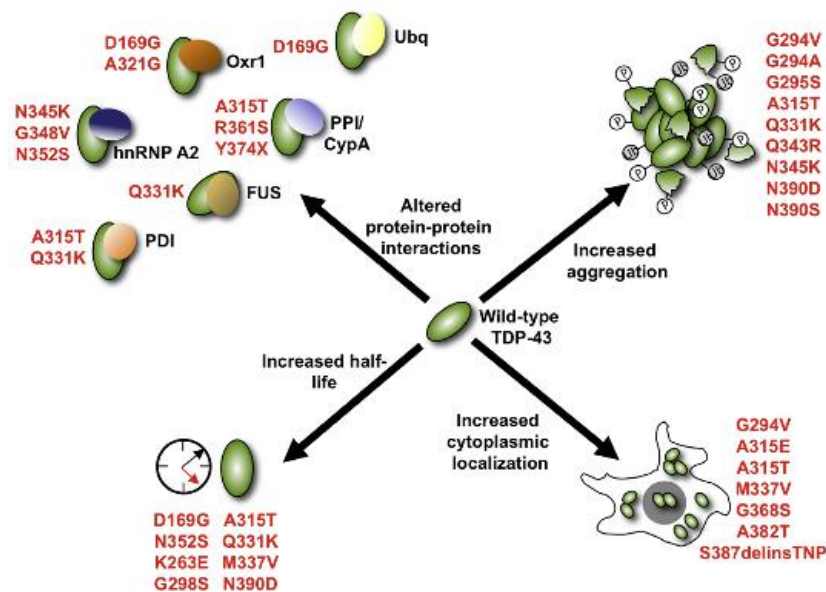
**Figure 19** TDP43 gene representation and localization of known mutations

TDP43 gene is composed of two RNA recognition motifs (yellow) and a glycine rich terminal region (red).

Most identified mutations causing ALS and FTD are located in the C-terminal glycine-rich-region (in red). Most of them are missense mutations. *Adapted from Lagier-Tournne (2013)*

Very few functional studies relating effects of *TARDBP* mutations to protein structure or function have been done. We now know some of the effects of each mutation (see Buratti 2015 for a detailed review). Briefly, the mutations can include altered protein interaction, increased half-life or increased aggregation, or higher protein mislocalization to the cytoplasm (Figure 20) (Buratti, 2015), but how these alterations will cause pathology has not yet been identified. In the following sections, the known implication of mutations will be described. As

progresses are being made, so is our understanding of *TARDBP* mutations in yielding toxicity, and soon, we may have a clearer picture of involved mechanisms.



**Figure 20** TDP43 mutations and their functional effects

TDP43 mutations can either cause altered protein-to-protein interaction, increased aggregation properties, increased half-life of increased cytoplasmic localization *Adapted from Buratti et al. (2015)*

### 1.2.2. TDP43 proteinopathies and aggregation

TDP43 proteinopathies were documented in a wide range of neurodegenerative diseases, including AD, PD and other tauopathies. The topology of TDP43 pathology can accord with symptomology, as is the case in ALS or FTD, or can have no association at all like in PD, in which the extend of pathology is limited both in amount and distribution. As variations in the composition of TDP43 aggregates between the different neurodegenerative diseases was observed, the formation of TDP43 pathology can either be directly correlated to pathological lesions as is the case for FTD or ALS, or be a secondary event as in these other diseases (Lagier-Tourenne et al., 2010). This observation raises the question of TDP43 mediated neurodegeneration, being a cause or a consequence of the pathology and whether abnormal TDP43 exacerbates degeneration (Chen-Plotkin et al., 2010; Lee et al., 2011). Of notice, TDP43 mRNA expression levels seem to be 1.5 fold elevated in patients with FTD and FTD/ALS when compared to controls (Buratti and Baralle, 2008). In iPSC models, increased cytoplasmic TDP43 levels were not accompanied by a depletion of nuclear TDP43

(Serio et al., 2013) suggesting that the loss of nuclear TDP43 arises as a later event in the disease process.

Furthermore, abnormally localized cytoplasmic TDP43 seemed to sequester normal endogenous TDP43 in vitro assays, further enhancing this mislocalization (Lee et al., 2011; Nonaka et al., 2013). In cases where no mutations of TDP43 were observed, abnormal pathological TDP43 is hyperphosphorylated, cleaved into 35kDa and 25kDa species, ubiquitylated, mislocalized and poorly soluble. Full length TDP43 has a half-life of 12-34h, whereas fragments have a half-life of ~4h (Lee et al., 2011). TDP43 mislocalization may stem from a translocation of TDP43 protein from the nucleus to the cytoplasm or from post-transcriptional modifications that prevent the crossing of the nuclear membrane by misfolded TDP43 (Buratti and Baralle, 2008; Davidson et al., 2007). Because the 25kDa fragment lacks the NLS, it is also possible that it accumulates in the cytoplasm by aggregation. The 25kDa fragment contains the C-terminal glycine-rich region that contains high levels of the amino-acid sequence glycine-glutamine-asparagine rich sequence. Such sequence exists in the prion-related domain of the TIA1-1 protein and has been shown to form specific aggregates and also mediate stress granules assembly (Wang et al., 2008). These aggregates might act as seeding sites for capture of newly synthesized TDP43 and other cytosolic factors. Conversely, in an *in vitro* model the 25kDa fragment formed cytoplasmic inclusions, induced cell toxicity, but did not disturb endogenous TDP43 functions. (Shan et al., 2010; Xu et al., 2010)

### 1.2.3. TDP43 hyperphosphorylation

A major site for TDP43 hyperphosphorylation is located at the Ser409/410 site, which is located right after the Arg408 proteolytic cleavage site producing the 25kDa fragment. This fragment is highly hyperphosphorylated and prominent in brains of ALS and FTD patients. Curiously, in transgenic mice, this fragment is rather located to the nucleus, whether in patients and in cell culture it is mainly cytoplasmic (Lagier-Tourenne et al., 2010). Mutating the serine residue 409 or 410 into alanines in mutant TDP43, and thus mimicking non-phosphorylated TDP43, eliminated its neurotoxicity in a *Caenorhabditis elegans* model (Lee, 2011). The 35kDa fragment is either low or undetectable in patients CNS, and in transgenic mice its expression decreases with disease progression. Rather than aggregating in a toxic form, the 35kDa TDP43 fragment may act as a partial isoform of the TDP43 protein. Evidence shows that the 35kDa fragment has a role in the formation of stress granules in cells under stress conditions (Colombrita et al., 2009; Nishimoto et al., 2010). It is unclear whether phosphorylation of TDP43 is an early event or rather an effect of earlier pathogenic events,

but it is clear that TDP43 phosphorylation is tightly correlated with its aggregation. Indeed, this post-translational modification is not necessary for C-terminal cleavage, aggregation or toxicity in *in vitro* models but a correlation between insolubility and phosphorylation has been reported (Chen-Plotkin et al., 2010; Lagier-Tourenne et al., 2010).

#### 1.2.4. TDP43 ubiquitylation

Ubiquitylation of TDP43 is seen at a later stage of disease progression. Early ‘pre-inclusions’ of TDP43 observed in human tissue are often non-ubiquitylated. At this point, neurons already exhibit decreased nuclear and increase cytoplasmic TDP43 immunoreactivity, which in turn, may contribute to increase the pools of TDP43 that can be latter ubiquitylated (Cairns et al., 2007; Igaz et al., 2009). TDP43 full-length protein and truncated fragments are degraded by the UPS (Igaz et al., 2009; Winton et al., 2008; Zhang et al., 2010). Inhibition of the UPS increases TDP43 aggregation in cultured cells (Eersel et al., 2011), suggesting a direct link between the UPS and TDP43 aggregation. Interestingly, mutations in ubiquilin 2 (*UBQL2*), a gene implicated in the protein degradation pathway, will cause ALS with TDP43 inclusions, further reinforcing the link of perturbations in the protein degradation pathway to aggregates formation (Lagier-Tourenne et al., 2010; Lee et al., 2011).

Autophagosome mediated degradation may also play a role in TDP43 turnover. TDP43 interacts with a variety of proteins involved in the autophagy pathway including the ESCRTs, ubiquilin 1 (*UBQLN*), SQMST /p62 and STAM binding protein (STAMBP). Depleting cells of ESCRTs, which are required for transporting proteins into multivesicular bodies, causes increased TDP43 aggregation and ubiquitylation (Filimonenko et al., 2007). Notably, mutations of the ESCRT subunit CHMP2B (charged multivesicular body protein 2B) causes FTD, although these cases do not exhibit TDP43 aggregation (Skibinski et al., 2005). Finally, mutations in *SQSTM1*, the gene coding for autophagy protein p62, have also been found as disease causing mutations for FTD (Fecto et al., 2011; Le Ber et al., 2013). Thus, the autophagy pathway seems to be strongly involved in FTD. These studies demonstrate a role for the UPS and autophagy systems in TDP43 turnover but they do not address whether these post-translational modifications are mechanistically involved in TDP43-mediated neurodegeneration.

#### 1.2.5. TDP43 mislocalization

Cellular localization of TDP43 aggregates varies between different diseases (see above and (Lagier-Tourenne et al., 2010), as inclusions can be intra-nuclear, cytoplasmic or be

present in dystrophic neurites. However, it is still unclear whether the different subcellular localizations of TDP43 protein rather reflect dysfunction of the cytoplasmic processing pathway or the nuclear import/export mechanisms in diseased cells. These observations have led to hypothesize two disease mechanisms: a gain of function effect caused by eventual toxicity of the aggregates themselves and a loss-of-function effect caused by the deprivation of TDP43 from the nucleus. These two mechanisms are not mutually exclusive and may both happen at the same time in the cell (Lee et al., 2011). Description of these two possible mechanisms is summarized in Figure 22 and corresponding section.

Genetic and environmental factors seem to influence TDP43 localization. TDP43 ALS-associated mutations near the NLS motifs (A90V) result in increased cytoplasmic expression, and disease-mutations in the *VCP* gene, which interacts genetically with TDP43 also results in redistribution from nucleus to cytoplasm. A variety of stressors can induce TDP43 relocation and colocalization with stress granules, as is the case of axotomy, which results in a time-dependent increase in cytosolic TDP43 expression in axotomized motor neurons (Lee et al., 2011; Moisse et al., 2009; Sato et al., 2009). The mislocalization of TDP43 differs from the observed nuclear clearance, as this clearance does not necessarily take place in the case of mislocalization of a nuclear protein (Lee et al., 2011), and different mechanisms might emerge from the presence of cytoplasmic versus nuclear TDP43 in disease.

### **1.2.6. TDP43 pathological models**

Several models of TDP43 pathology present contradictory results, making it complex to understand the key question of mutant TDP43-mediated pathogenesis. Homozygous disruption of the *TARDBP* gene is embryonically lethal, but heterozygous gene disruption does not cause developmental problems, since protein levels are identical to wild-type levels. An alternative approach to study the effects of reduced TDP-43 expression is to use a conditional knockout strategy. Conditional mouse models showed that homozygous deletion of *TARDBP* caused rapid loss of body fat and death within 9 days (Chiang et al., 2010) and depleting Hb9-positive spinal cord motor neurons of TDP-43 resulted in significantly reduced bodyweight at 8 weeks of age (Shan et al., 2010). These animals developed Rotarod deficits by 15 weeks; followed by abnormal hind-limb clasping and kyphosis and motor neuron loss was accompanied by age-dependent gliosis and cytoplasmic aggregation of ubiquitin in motor neurons. A sex-bias towards males accompanied development of phenotypes in these animals

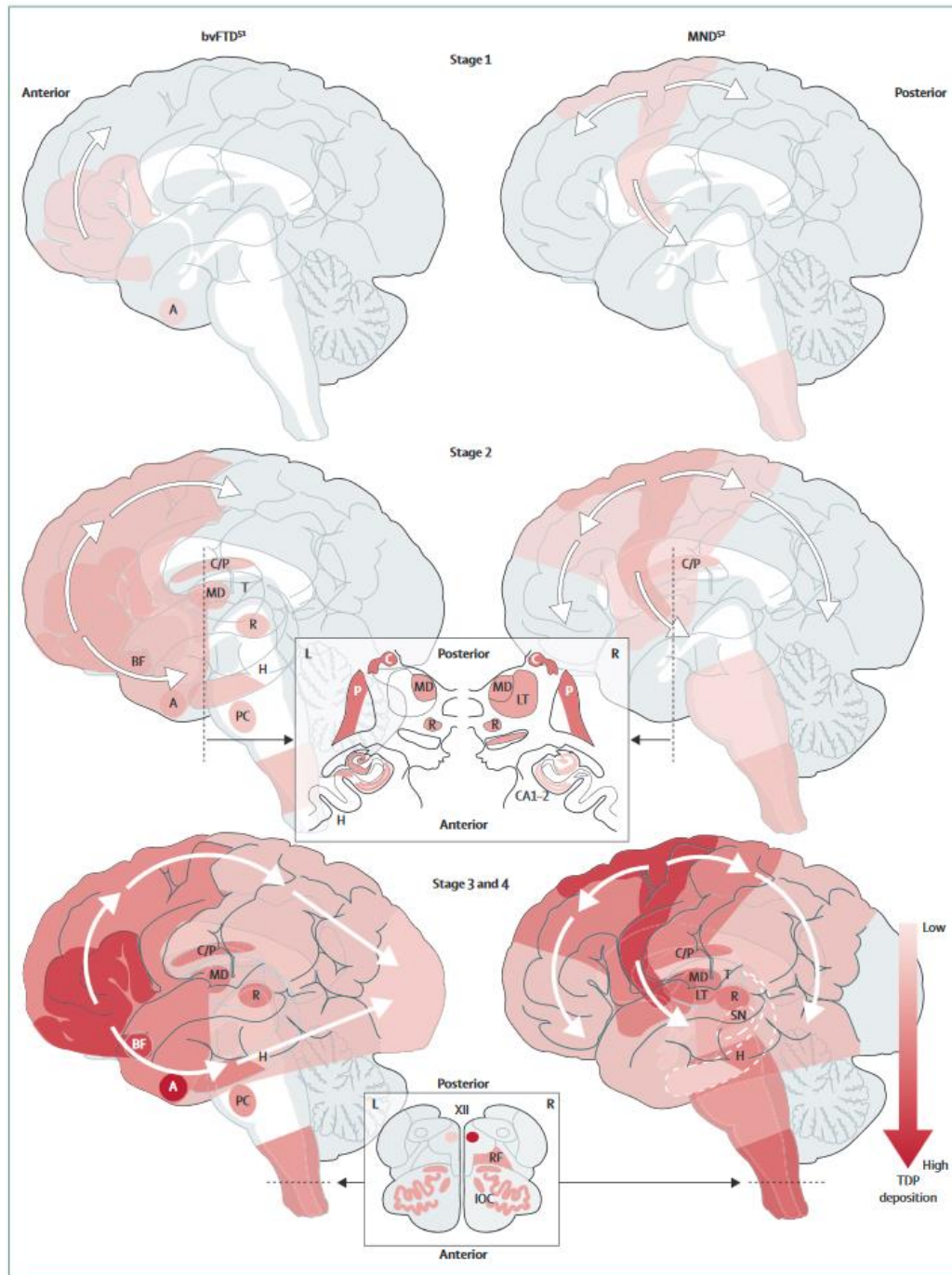


with average lifespan being approximately 10 months. Similar observations arise in *Drosophila* models in which surviving null-flies display locomotion deficits and defects in neuromuscular junction and reduced lifespan (Li et al., 2010), as well as axonal loss and neuronal death (Feiguin et al., 2009).

Expression of wild-type human TDP43 in *Drosophila* and rodent models causes neurodegeneration associated with impaired locomotion activity, paralysis and reduced lifespan, (Cohen et al., 2011; Igaz et al., 2011; Lee et al., 2011; Xu et al., 2010). However, development of disease phenotypes seems highly dependent upon the promoter used and the level of transgene expression (McGoldrick et al., 2013). Expression of mutant TDP43 causes increased toxicity in chick embryos and a motor phenotype in zebra fish (Kabashi et al., 2010; Tripathi et al., 2014). Mice carrying a common TDP43 mutation (A315T) presented gait abnormalities consistent with upper motor neuron disease. Loss of cortical layer V pyramidal neurons in frontal cortex has been observed with increased microgliosis and astrogliosis in both mutant and wild-type mouse models, suggesting a selective vulnerability of this type of neurons (McGoldrick et al., 2013; Wegorzewska et al., 2009). Mice models have shown early TDP43 pathology, especially when overexpressed, but a direct relation between the number of inclusions and degeneration has not been observed (Lee et al., 2011; McGoldrick et al., 2013). On the other hand, TDP43 transgenic mice expressed C-terminal fragments (CTFs) before development of gait abnormalities or neurodegeneration, suggesting that CTFs may have a primary role in causing toxicity (Wegorzewska et al., 2009). However, in these mice models, CTFs are found more often and in a more soluble state than what is observed in patients. Furthermore, not all mouse models express CTFs despite having similar altered phenotypes and the role of CTFs has yet to be described in the absence of full-length protein, thus questioning the validity of the related toxicity. An approach that compares the relation between levels of expression (high or low) to the actual phenotype might be more suitable for identifying actual pathogenic mechanisms of these CTFs. Few studies have addressed the role of CTFs, and up to date it is known that they impair neurite growth during differentiation of cultured neurons, but they do not cause neuronal toxicity in *Drosophila* or chick motor neurons. Finally, several TDP43 transgenic mice models have TDP43 negative cytoplasmic inclusions composed of mitochondrial aggregates that are not seen in patients (Lee et al., 2011). As previously presented, one of TDP43 functions may raise in stress granule formation, so TDP43 cytoplasmic accumulation may represent the typical response to stress rather than only an initiating event in pathogenesis.

### 1.2.7. TDP43 mediated neurodegeneration

Unresolved controversy remains on whether inclusions are neurotoxic by binding essential proteins or neuroprotective by creating inactive species of toxic protein forms, as findings pile up supporting both observations. Combined data from experimental models have showed that TDP43 aggregates themselves will not cause neurodegeneration, but they will contribute to neuronal dysfunction and death. The presence of a prion-like domain in TDP43 has raised its possibility spread disease from cell-to-cell, particularly since spread through contiguously motor neurons has been described in ALS patients (Furukawa et al., 2011; Smethurst et al., 2015). Evidence of pathological infiltration shows a preferential directionality along the motor tracks, advancing in sequential stages (**Figure 21**) (Burrell et al., 2016). On the contrary, observations showing that in some cases progression is noncontiguous points rather to a multifocal hit and local propagation of disease have also been described (Smethurst et al., 2015). Regardless of these observations, similarities between a prion-like propagation and TDP43 pathology are compelling. Mutant and wild-type TDP43 are capable of seeding properties and of propagating between cells, (Furukawa et al., 2011; Nonaka et al., 2013) but this effect has yet to be demonstrated *in vivo* studies. Mechanisms explaining cellular propagation have not been clarified yet, but it is possible that the aggregates fragment and when released in neighbor cells or transported through axons to the synapse they will be uptake in the next cell (Smethurst et al., 2015). It has thus become essential to understand anatomic spread and clinical progression of TDP43 pathology.

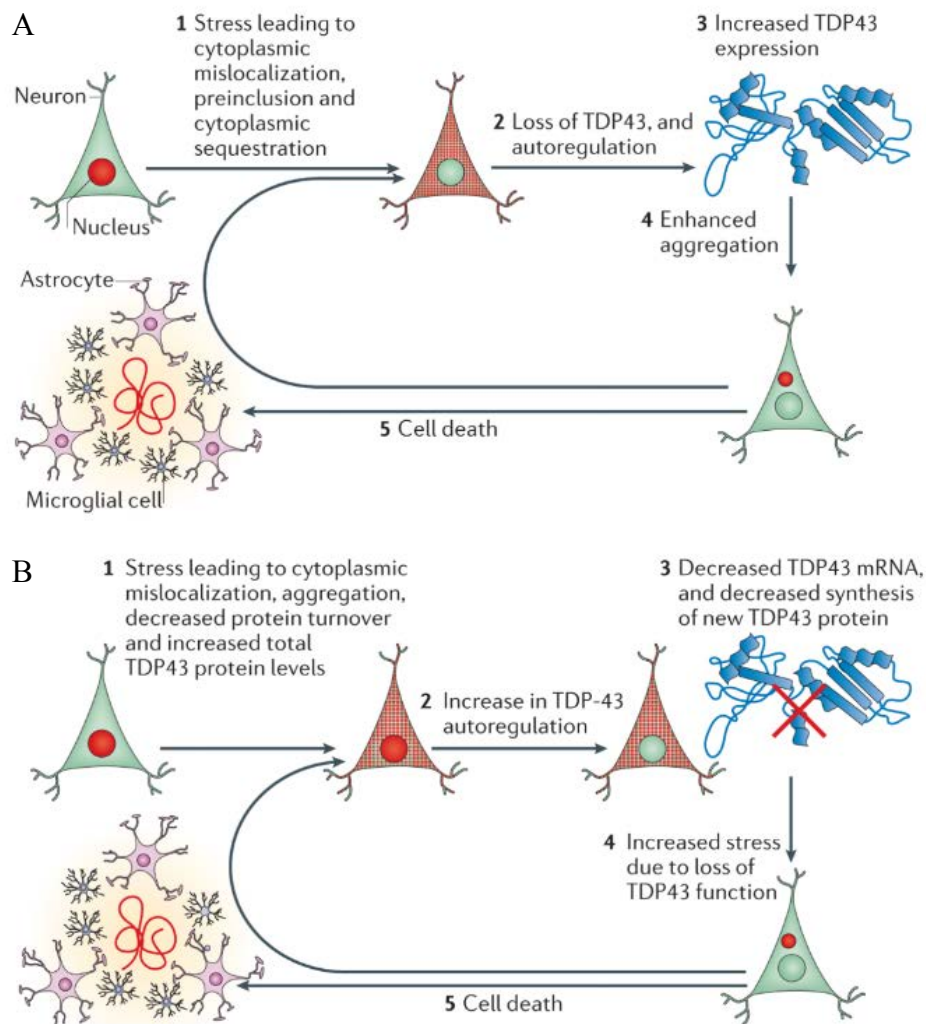


**Figure 21** Stages of TDP43 spreading and deposition in FTD (left) and ALS (right)

TDP43 pathology in appears to start from the agranular frontal neocortex and somato-motor neurons of the spinal cord and lower brain stem, eventually spreading to the medial temporal lobe and hippocampus by the latest stage *From Burrell et al. (2016)*

Multiple pathways are likely to be involved in TDP43 mediated neurodegeneration. Given the plethora of TDP43 functions, its mislocalization to the cytoplasm could cause a loss of normal nuclear functions and thus weaken the cell or eventually cause its death. Additionally, its presence in the cytoplasm could cause a toxic gain of function by its RNA-binding properties. Or even maybe, CTFs could act as toxic molecules by binding other hnRNP sites without bringing the usual RNA cargo. By the wide range of information the literature has provided, a (1) loss of autoregulation model and a (2) gain of autoregulation model have been proposed (**Figure 22**) (Lee et al., 2011). The first one proposes that initial abnormal toxic RNA species will induce cellular stress and thus cause redistribution of TDP43 from the nucleus to the cytoplasm. This can either cause the formation of stress granules or the abnormal distribution of RNA transport granules. This redistribution will result in the aggregation of phosphorylated and ubiquitylated TDP43. As disease course advances, more TDP43 will be redistributed from the nucleus accounting for nuclear clearance. This lack of TDP43 in the nucleus will cause autoregulation of TDP43 expression leading to increased aggregate formation and into a vicious circle that will eventually lead to cell death (Lee et al., 2011). The second model proposes that a stressor will lead to TDP43 mislocalization resulting in aggregation. If autoregulation of TDP43 takes place in the cytoplasm, TDP43 aggregates will retain its ability to bind its mRNA and therefore autoregulate it in a negative loop decreasing TDP43 synthesis. This will eventually lead to nuclear clearance and a loss-of-function of nuclear TDP43 increasing stress and leading to cell death (Lee et al., 2011).

Whether any of these models of autoregulation takes place in affected human brains has not been demonstrated yet, but as neurodegeneration correlates better with TDP43 nuclear clearance in transgenic mice than with post-translational modifications, aggregation or subcellular localization, great interest should be taken as it this deregulation is probably linked to neuronal loss (Igaz et al., 2011).



**Figure 22** TDP43 toxicity models

(a) Loss of autoregulation model proposes TDP43 mislocalization due to exposure to an unknown stressor factor leading to increased TDP43 expression and enhanced aggregation (b) Gain of autoregulation model proposes that cytoplasmic mislocalization will cause increase in TDP43 autoregulation creating a toxic loop due to TDP43 loss-of-function *From Lee et al. (2011)*

These two proposed models highlight the possibility that gain and loss-of-function alterations may together affect neuronal viability. As TDP43 pathology is present in most cases of ALS and FTD, it has a central role in pathogenesis, and thus understanding its function in RNA metabolism is crucial. Much remains yet to be discovered to be able to propose treatments to patients.

Recently, an *in vitro* model showed that TDP43 could directly bind mRNA containing G-quadruplex (Ishiguro et al., 2016). As TDP43, through its RNA binding domain, can act as a transport molecule (Buratti and Baralle, 2010), the authors propose that TDP43 acts as a vehicle for carrying mRNA to neurites and other subcellular domains for local translation.

When exposed to high levels of G-quadruplex structures, as would be the case in C9FTD/ALS, TDP43 will be sequestered in the excessive forms causing depletion of TDP43 functional pool. This result, although interesting, is controversial. Characterization of patient brains and spinal cords inclusions has not revealed colocalization of TDP43 with RNA foci, and so far, the result has not been replicated.

Thus, the link between TDP43 pathology and the C9orf72 repeat expansion has not been established yet. Identifying this link will be a great advance to the community given that both are highly implicated in FTD/ALS.

## 2. C9orf72 in ALS and FTD physiopathology

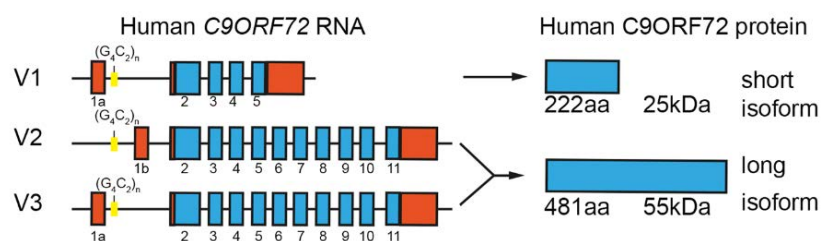
The discovery in 2011 of the GGGGCC hexanucleotide expansion as the most common cause of both ALS and FTD caused great commotion in the scientific community. Over the past 5 years, a number of possible mechanisms leading to neurotoxicity have been identified, without fully elucidating the trigger event(s) that leads to neuronal death and to the clinical phenotype.

### 2.1. C9orf72 gene and protein

Located on chromosome 9, the gene comprises 10 coding and two non-coding exons (1a and 1b) that generate three protein-coding mRNA transcript variants (variant 1 exon 2-5, variant 2 and variant 3 exon 2-11) resulting in two protein isoforms of 222 aa (C9-S) and a 481 aa (C9-L) respectively (**Figure 23**). Two more sense transcript and 3 anti-sense transcript in the CNS have been recently described; but their presence does not seem to alter protein composition (Rizzu et al., 2016). Initial analysis of C9orf72 normal expression showed it to be of around 80% in the sense strand and of 20% in the antisense strand, with the highest expression levels being in neurons, especially in the cerebellum, frontal cortex, hippocampus, hypothalamus and spinal cord. The major-predicted form is the V2 transcript (DeJesus-Hernandez et al., 2011; Renton et al., 2011; Suzuki et al., 2013). More recently, a group published that C9orf72 expression is very high in a subset of myeloid cells involved in innate and adaptive immunity including CD14<sup>+</sup> monocytes, but also eosinophils, and neutrophils (Rizzu et al., 2016). Suzuki and colleagues identified that the mouse orthologue of C9orf72 was poorly expressed in spinal microglia and astrocytes, and Rizzu and colleagues found that human macrophages themselves had a rather low level of C9orf72 expression, whereas precursor cells presented a high level of expression. However, other teams argue that microglia has the

highest levels of *C9orf72* expression in the CNS (O'Rourke et al., 2016; Y. Zhang et al., 2014), but validation in human samples are still missing. Interestingly, challenging monocytes with pathogens will cause a significant increase in *C9orf72* expression and complete ablation of *C9orf72* in mice models causes immunological deficits, suggesting an important role related to myeloid function and monocyte maturation, regardless of its expression levels (Atanasio et al., 2016; O'Rourke et al., 2016; Rizzu et al., 2016).

To date, the role of the *C9orf72* protein remains largely unknown. Bioinformatics studies have predicted it to belong to the DENN protein family. Proteins with DENN domains are known to act as Rabs Guanosine Exchange Factors (Rab-GEFs) that activate Rab-GTPases and hence regulate membrane trafficking and vesicular formation (Levine et al., 2013; Zhang et al., 2012). Characterization of the protein has been challenging, since commercial antibodies offer poor sensitivity and specificity, leading to results that were not often replicated between papers. However, several groups have tried to produce in house antibodies and initial results of *C9orf72* protein localization are starting to emerge. It has been shown that the two *C9orf72* protein isoforms have distinct biochemical profiles and present different subcellular localizations (**Figure 23**) (Atkinson et al., 2015). The C9-L isoform presents cytoplasmic staining in neurons and cerebellar Purkinje cells, whereas the C9-S isoform colocalize with nuclear membrane proteins, and more precisely, with components of the nuclear pore complex. Furthermore, a group showed that *C9orf72* interacts with several Rabs, including the endocytic Rab5 and Rab7 and by this interaction, regulates membrane trafficking in neuronal cell lines and human spinal cord motor neurons (Farg et al., 2014). In the same study, it was shown that *C9orf72* interacts with Rab1, RAB11 and the LC3 domains of autophagosome structures, ubiquilin-2 hnRNPA1 and hnRNP A2/B1, further linking its function to autophagy and cellular trafficking, but also to RNA metabolism. In the same line, proteomic analysis revealed that *C9orf72* interacts with SMCR8 and WDR41 proteins to form



**Figure 23** *C9orf72* gene and protein isoforms

*Adapted from Atkinson et al. (2015)*

a GDP/GTP exchange structure for Rab GTPases involved in autophagy (Ciura et al., 2016). In this study, decreased levels of C9orf72 in cultured neurons led to accumulation of p62 aggregates and phosphorylated TDP43 without major neuronal toxicity. Additionally, bone-marrow-derive-macrophages from knockout mouse models of C9orf72 presented a defect in late-endosomal trafficking (O'Rourke et al., 2016). These animals presented marked accumulation of Lyso-Tracker and Lamp1-positive vesicles but no changes in Rab5 and Rab7 were observed. Interestingly, when expressing human C9orf72, this alteration was rescued in macrophages, indicating a direct link between the loss of C9orf72 and the alterations in the phagosomes-to-lysosomes maturation pathway. All these findings suggest that decreased expression of C9orf72 might dysregulate autophagy/endosomal trafficking pathways and contribute or modulate disease pathogenesis.

## **2.2. Pathological C9orf72**

The GGGGCC (G<sub>4</sub>C<sub>2</sub>)<sub>n</sub> HRE lies within an intronic portion of the gene, located either in the promotor region of transcript V2 or within the first intron of transcripts V1 and V3 (Figure 23). As was briefly described in Chapter II and in Figure 13, the expansion could lead to pathology through three different mechanisms: (a) RNA foci sequestering of RBPs depleting the cell of these proteins (b) DPR proteins forming intracellular inclusions disturbing neuronal function and (c) protein loss-of-functions impairing autophagy and endocytosis at least. In this section I will present knowledge of these three mechanisms.

### **2.2.1. Repeat-expansion toxic gain-of-function contribution to disease pathogenesis**

#### **2.2.1.1. RNA toxicity**

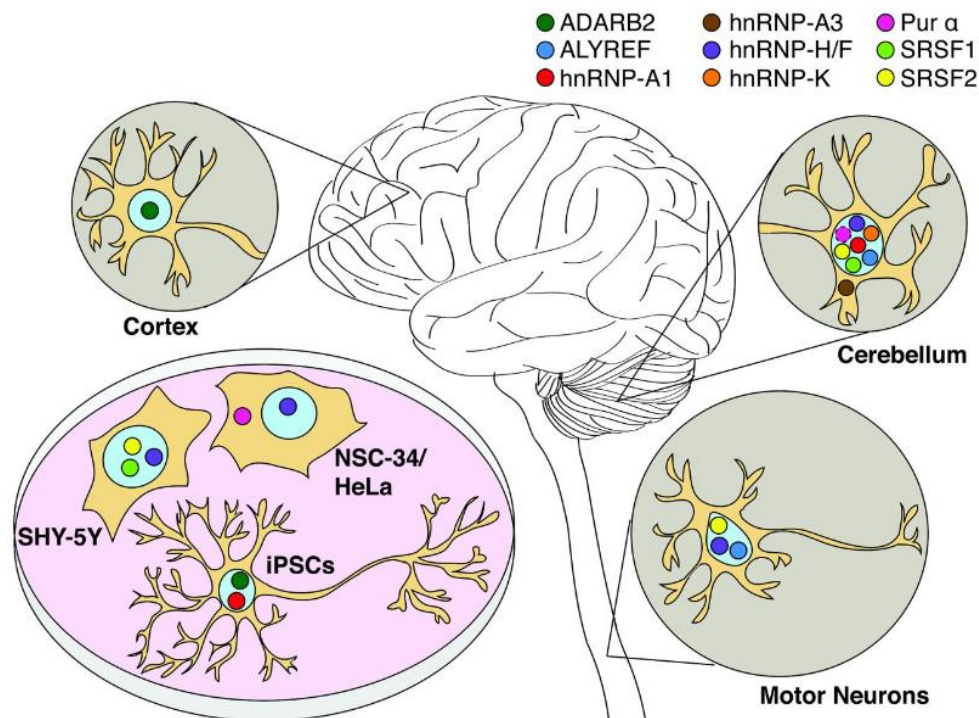
The C9orf72 HRE expansion can form stable DNA and RNA G-quadruplex structures, and less stable hairpin structures, existing in an equilibrium between these two forms (Fratta et al., 2012; Haeusler et al., 2014). Observations of cellular expression of either sense or antisense RNA transcripts leading to the presence of nuclear RNA foci have been found in cerebellum, motor and frontal cortex, hippocampus, interneurons, glia and spinal cord of C9orf72 expansion carriers (Cooper-Knock et al., 2015b; DeJesus-Hernandez et al., 2011; Donnelly et al., 2013; Lagier-Tourenne et al., 2013; Mizielinska et al., 2014, 2013; Zu et al., 2013). Numerous localizations of RNA foci throughout the CNS have been found (Cooper-Knock et al., 2015b; Mizielinska et al., 2013). Furthermore, the same studies did not agreed



about in TDP43 localization or the presence of p62 inclusions making it hard to determine whether the foci themselves can cause cellular dysfunction or they are just a consequence of protective cellular mechanisms (Haeusler et al., 2014).

RNA toxicity has been proposed as a possible pathogenic mechanism, as RNA foci sequester RBPs depleting the cell of their function. This type of pathological mechanism has been described in myotonic dystrophy (Wojciechowska and Krzyzosiak, 2011). Studies have identified colocalization of several hnRNPs -H, A1, A2/B1, A3-, Pur alpha, ADARB2 (adenosine deaminase RNA specific), RanGAP with C9orf72 RNA foci in *in vitro* models (Figure 24) (Cooper-Knock et al., 2015b, 2014; Donnelly et al., 2013; Haeusler et al., 2014; Lee et al., 2013; Sareen et al., 2013; Xu et al., 2013; Zhang et al., 2015). But evidence backing their altered expression in patients is lacking and no colocalization has been observed in animals models presenting RNA foci (Jiang et al., 2016; Liu et al., 2016; O'Rourke et al., 2015; Peters et al., 2015). Interestingly, a group found that nucleolin had an altered localization (O'Rourke et al., 2015). Nucleolin can bind C9orf72 RNA G-quadruplex, and by this binding cause nuclear stress (Haeusler et al., 2014).

In parallel, hnRNP A3 is relocalized to the cytoplasm where it colocalize with p62-positive, TDP43 negative inclusions (Mori et al., 2013a) but does not colocalize with nuclear



**Figure 24** RBPs and the C9orf72 repeat expansion

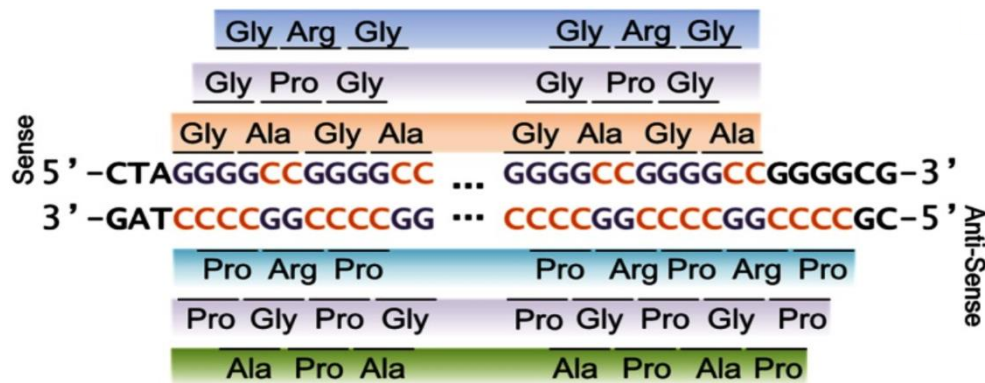
Localization of different RBPs identified as colocalizing with the C9orf72 repeat expansion *Adapted from Todd and Petrucelli (2016)*

RNA foci in patients cerebellum or iPSC-derived motor neurons (Lee et al., 2013; Sareen et al., 2013). These findings may suggest that hnRNP A3 plays a role in shuttling of HRE-mRNA from nucleus to cytoplasm where it undergoes RAN translation. Pur alpha nuclear inclusions were detected in cerebellar granular cell of C9FTD/ALS patients (Xu et al., 2013) and colocalization with RNA foci was seen in iPSC-derived motor neurons (Sareen et al., 2013) but not in SH-SY5Y cells (Lee et al., 2013). In this last study, colocalization of hnRNP H with foci was also shown, with hnRNP H subtracts having altered alternative splicing (Lee et al., 2013; Rossi et al., 2015). As for Pur alpha, ADARBP is found to co-localize in iPSC-derived motor neurons and in the motor cortex of C9ALS patients. Diminishing ADARBP levels reduces the number of RNA foci, suggesting it may be important for their formation or maintenance (Donnelly et al., 2013). Finally, binding of RanGAP with C9orf72 as does the mRNA export factor AlyRef causes mislocalization of these proteins to the cytoplasm which seems to cause disruption of nucleocytoplasmic transport (Cooper-Knock et al., 2014; Zhang et al., 2015).

In summary, C9-HRE can bind several RBPs. Whereas this sequestering prevents normal function of protein and thus leads to disease pathogenesis remains yet to be demonstrated, as in most studies no functional analyses have been performed. Furthermore, there is an important variation in the interacting proteins identified among groups without reiteration in other models or in patient tissues. Of great interest, a  $(G_4C_2)_{160}$  *Drosophila* model that expressed high levels of sense RNA foci in the nucleus with low levels of RAN translation did not developed eye degeneration (Tran et al., 2015), indicating that RNA foci in the nucleus do not contribute to neurodegeneration in fly models. These results have yet to be reproduced in human cells and mouse models.

#### **2.2.1.2. Dipeptide Repeats Toxicity**

In microsatellite-expansion diseases, mutations localization can cause two well-known effects. Taking the example of HD, the mutated expansion located within the coding exon will produce a pathogenic highly aggregating polyglutamine (polyGln) tract and when the mutated expansion it is located outside the coding exon as is the case in myotonic dystrophy, toxicity will be due to toxic RNA transcripts that capture RBPs (Wojciechowska and Krzyzosiak, 2011). In 2011, Zu and colleagues found a non-canonical form of translation where mutated expansions are translated in 3 phases into small-aggregating proteins without the need of an AUG-initiation codon (Zu et al., 2011). This form of translation is known as repeat associated non-ATG (RAN) translation and the translated products are known as dipeptide repeat



**Figure 25** RAN translation products of the C9orf72 repeat expansion

Schematic representation of the proteins generated by RAN translation from the GGGGCC expanded repeat of the *C9orf72* gene  
*Adapted from Gendron et al. (2013)*

proteins (DPRs). The position of the C9orf72 HRE mutation, outside the ATG-initiated open reading frame (ORF) leads to the production of DPRs in patient brains and spinal cords (Ash et al., 2013; Mackenzie et al., 2013, 2015; Mori et al., 2013b; Zu et al., 2013).

In c9FTD/ALS, the sense strand encodes three dipeptide proteins GlyPro (GP), GlyArg (GR) and GlyAla (GA), whereas the antisense strand encodes for ProGly (PG), ProArg (PR) and ProAla (PA) (**Figure 25**) that accumulate in neuronal inclusions in the cerebellum, hippocampus, amygdala, medulla, thalamus, frontal and motor cortex and spinal cord of patients (Mackenzie et al., 2013; Zu et al., 2013). Initially, localization of DPRs to regions dissociated from clinical aspects of FTD or ALS raised the question of DPR significance to pathology, and called for caution in considering DPRs as primary pathogenic agents (Davidson et al., 2014; Gomez-Deza et al., 2015; Mackenzie et al., 2013, 2015, 2014; Prudencio et al., 2015; Todd and Petrucelli, 2016). However, some studies have found a relation between DPRs and clinical presentation of FTD, showing a correlation in cognitive dysfunction with DPR pathology (Gendron et al., 2015; Schludi et al., 2015). Furthermore, cerebellar and thalamic atrophy have been observed in c9FTD/ALS patients through multiple imagery studies (Mahoney et al., 2012; Schipper et al., 2015; Sha et al., 2012; Whitwell et al., 2012). Even though correlative studies give insight into DPRs toxicity, their effects on cellular types have yet to be defined. Of particular interest, as TDP43 mislocalization does not correlate with DPRs, it has been suggested that DPR pathology might precede TDP43 pathology (Baborie et al., 2015; Davidson et al., 2014; Mackenzie et al., 2013; Proudfoot et al., 2014; Schipper et al., 2015). This seems to be the case for an asymptomatic short



in patient brains suggest DPRs interaction with the proteasome or the autophagy pathway (Mackenzie et al., 2013). In *Drosophila* models, poly (GA) does not cause toxicity (Freibaum et al., 2015; Mizielinska et al., 2014; Yang et al., 2015) but it is neurotoxic in mice (Zhang et al., 2016) (*discussed below*). The exact role of DPRs in c9FTD/ALS remains therefore to be understood, and caution should be taken as different models present different, sometimes opposite effects. Furthermore, it could be that different DPRs could have different effects in different cell types, complicating even more our understanding of mechanisms that will trigger or drive disease (**Figure 26**).

### **2.2.1.3. Insights into gain-of-function toxicity from models**

Neurotoxicity caused by RNA foci or DPRs remains highly debated, as dissociation between the two mechanisms has rarely been possible. The development of disease models gives insight into the toxicity caused by the expansion, but results remain difficult to interpret. Induced-pluripotent-stem cells (iPSC) models have been a great tool to study C9orf72 associated pathology as they present both RNA foci and DPRs pathology and have decreased C9orf72 expression. Nevertheless, in this model dissociation of toxicity between mechanisms has not been possible. iPSCs derived cortical neurons present enhanced sensitivity to autophagy (Almeida et al., 2013) whereas derived motor neurons are extremely sensitive to endoplasmic reticulum stressors, to extracellular glutamate stimulation and present abnormalities of axonal excitability (Devlin et al., 2015; Donnelly et al., 2013; Geevasinga et al., 2015; Haeusler et al., 2014). In accordance with this observation, cortical hyperactivity was identified in ALS patients carrying the C9orf72 expansion, but not in FTD or ALS/FTD patients (Schanz et al., 2016).

Development of *Drosophila melanogaster* models that reproduce RNA toxicity, DPRs toxicity or both have allowed for a better discernment between RNA-toxicity gain-of-function and DPR pathology (Boeynaems et al., 2016; Mizielinska et al., 2014; Tran et al., 2015; Xu et al., 2013). A initial report presented that expression of (G<sub>4</sub>C<sub>2</sub>)<sub>30</sub> in the fly eye or motor neurons caused progressive neurodegeneration and locomotor activity dysfunction through RNA-toxicity alone (Xu et al., 2013). Contrariwise, two separate groups have shown that RNA toxicity only is not enough to drive neurodegeneration in the fly, and that arginine-rich DPRs drive degeneration (Mizielinska et al., 2014; Tran et al., 2015). Experimental data have shown DPR toxicity, but no link to disease onset has been observed so far. Toxicity led by DPRs would suggest an enhanced accumulation in affected regions. Postmortem analysis from c9FTD/ALS patients have not shown a correlation between DPRs localization,

neurodegeneration or clinical presentation (Davidson et al., 2014; Mackenzie et al., 2015, 2014; Schludi et al., 2015). In brain samples, poly(GA) and poly(GP) appear to be the most abundant, with poly(GR) being less present. Antisense-transcripts DPRs are exceptionally rare (Mackenzie et al., 2015)

The development of mice models has given important understanding of the pathology caused by gain-of-function mechanisms. In the last year, a myriad of mice models have been published giving insight into *C9orf72* HRE toxicity. The first model reproducing *C9orf72* pathology was created using a viral delivery approach. Expression of (G<sub>4</sub>C<sub>2</sub>)<sub>66</sub> in CNS of mice caused behavioral and motor alterations, TDP43 mislocalization, presence of nuclear foci, DPR pathology and neuronal loss (Chew et al., 2015). Shortly after, using a similar viral approach, a mouse model exhibiting poly(GA) pathology only was reported. These animals developed cognitive, memory and motor dysfunction with p62 and ubiquitin-positive inclusions in the absence of TDP43 pathology (Zhang et al., 2016). These two models mimicked key hallmarks of *C9orf72* pathology, but high levels of RNA and DPR expression raised questions into their congruity.

At the same time, two groups used bacterial artificial chromosome (BAC) to express part (exons 1-6 with 140kb 5' flanking region) (Peters et al., 2015) or all (110kb 5' and 3' 20kb flanking regions) (O'Rourke et al., 2015) of the human *C9ORF72* gene in transgenic mouse models. Contrary to the AAV2-viral models, these animals did not developed degenerative processes including TDP43 mislocalization and neuronal loss, even in the presence of DPR pathology and RNA foci. These observations raised the question of the effects of abnormal expression of the HRE rather that expression of physiological levels and how do these will trigger or drive neurodegeneration. A parallel hypothesis was the possibility that the toxicity-levels needed to cause degeneration need to be presented chronically in a timeframe that exceed the lifespan of laboratory mice.

Now (in May 2016), two groups published new BAC transgenic mice using patient-derived gene constructs, either using a construct containing *c9* exons 1-5 (140kb 5' flanking region) (Jiang et al., 2016) or full *C9orf72* gene including 5' and 3' flanking regions (Liu et al., 2016). These new models develop molecular, neuropathological and clinical features of *c9*FTD/ALS. The mice reported by Jiang and colleagues developed DPR pathology and presence of RNA foci throughout the CNS. They did not show evidence of neuronal or motor neuron loss, altered motor function, gliosis or TDP43 mislocalization, although increased levels of phosphorylated TDP43 were quantified by western-blot. Nuclear pore structure seemed conserved as no alteration of RanGAP or lamin  $\beta$  was seen. These mice developed

cognitive deficits by 12 months of age including spatial learning and memory deficits (Barnes maze and Radial arm maze) and anxiety like behavior (Elevated plus maze and Marble burying tests), but no behavioral alterations in social interaction, social recognition or social communication (novel object recognition, fear conditioning and serial reversal learning). By comparison, Liu and colleagues reported 4 lines of transgenic mice with different lengths of the HRE presenting RNA foci and DPR pathology. Authors report that DPR proteins were challenging to identify and only poly(GA) and poly(GP) were found. A subset of female animals from the highest repeat-line (500 repeats) developed sudden and rapidly progressive weight loss, difficulty in breathing and death at 12 months of age, with neuromuscular junction denervation, muscle atrophy and TDP43 mislocalization. The remaining animals developed a slow progressive phenotype with gait abnormalities (grip strength, open field, treadmill) and anxiety like behavior (open field) between 52-72 weeks of age with mild neuromuscular junction alterations. A really interesting observation is the fact that antisense but not sense foci correlated with the severity of the degeneration, which is in accordance with previous reports where no degeneration was observed with sense RNA foci (Tran et al., 2015).

Overall, knowledge obtained from the different gain-of-function models determines that RNA foci and DPRs can be toxic in a dose-dependent manner, as in most cases overexpression was used, and at physiological levels, the effects may be milder as is the case in the C9orf72 BAC transgenic mice. Nevertheless, it is most probable that both DPRs and RNA foci toxicities co-occur in the cell, causing a joint toxicity in several concomitant pathways to eventually cause degeneration and death.

Several other models have provided information of cellular mechanisms driving pathogenesis. Although several pathways seem to be implicated (**Table 4**), the two main processes implicate nucleolar stress and nucleocytoplasmic shuttling abnormalities.

### **2.2.2. C9orf72 loss-of-function contributions to disease pathogenesis**

Altered C9orf72 function (i.e. haploinsufficiency) has been considered as a possible pathomechanism due to the observation that expression of C9orf72 is reduced in patient tissues (Blitterswijk et al., 2015; DeJesus-Hernandez et al., 2011; van der Zee et al., 2013). Quantification of all transcripts has shown an overall reduction of expression with decreased expression of transcripts variant 1 and 2 principally in the cortex with a lower decrease in the cerebellum and almost not identified or minor variation of transcript 3 (Blitterswijk et al., 2015; Xiao et al., 2015). Unexpectedly, a correlation between transcript variant 1 levels and

prolonged survival has been observed after disease onset (Blitterswijk et al., 2015). This transcript variant codes for the short protein isoform, which localizes to the nuclear membrane, suggesting a possible beneficial effect of conserving its function. This observation raises great interest, since several groups have described nucleocytoplasmic trafficking impairment in C9FTD/ALS (Boeynaems et al., 2016; Freibaum et al., 2015; Jovičić et al., 2015; Zhang et al., 2015, 2016). Further support for this idea comes from the observed interaction of C9orf72 with nuclear pore complex (NPC) components and the fact that this interaction correlated inversely with cytoplasmic levels of TDP43 protein (Xiao et al., 2015). Further research will soon arise looking into the beneficial effects of preserving this nuclear function, particularly if TDP43 mislocalization is linked to a possible function of C9orf72 in nucleocytoplasmic shuttling.

Initially hypothesized, evidence is now showing that the HRE can generate C9orf72 truncated transcripts that contain the expansion (Blitterswijk et al., 2015; Haeusler et al., 2014). These truncated transcripts can aggregate creating RNA foci. The HRE also causes shift of transcription from exon 1b (V2) to exon 1a (V1 and V3) (Sareen et al., 2013), possibly explaining the decrease of transcript V2 in patient brains. Reduced promoter activity has also been observed in expansion carriers (which is dependent on expansion size) (van der Zee et al., 2013). Furthermore, epigenetic modifications are capable of altering expression, most particularly hypermethylation of the CpG island upstream of the promoter (Liu et al., 2014; Russ et al., 2015; Xi et al., 2013). The effect of these modifications remains controversial as they seem to have a protective role in certain studies (Bauer, 2016; Liu et al., 2014) but cause diminished disease duration in other cases (Xi et al., 2013). Gene silencing further occurs through the binding to trimethylated histones, and this phenomenon has also been observed in C9orf72 cases

Even though, protein levels have been less studied, a decreased level of C9-L isoform in the temporal and frontal cortices of C9orf72 HRE carriers has also been quantified (Waite et al., 2014; Xiao et al., 2015) which supports a haploinsufficiency effect. Xiao and colleagues (2015) did not find a difference in motor cortex and cerebellum and detection of the long isoform was extremely low in spinal cords. Nuclear membrane labeling of C9-isoforms was diminished or completely absent in ALS, as was Importin  $\beta$  labeling but not laminin staining, suggesting that C9orf72 may be implicated in nuclear import. Conversely, a homozygous case for C9orf72 HRE did not present an enhanced clinical phenotype, challenging the idea that the loss-of-function drives disease pathology (Fratta et al., 2013). In this case study, protein quantification was not performed and since the mutation is located in a



non-coding region it will not directly affect protein formation, thus a basal protein level could remain in this patient.

Interestingly, decreased levels of *C9orf72* were shown in FTD patients with mutations in *MAPT* and *GRN* genes in frontal and temporal cortices and in hippocampus, suggesting that observed reductions of *C9orf72* are not uniquely dependent on the HRE expansion, and that other molecular mechanisms influence its expression (Rizzu et al., 2016). As this variation was not seen in other neurodegenerative diseases such as PD and AD, a specific FTD process seems to cause this decrease. Studies that corroborate this observation are still needed. Analysis of expression levels in human neuroblastoma cells showed a possible molecular pathway of interaction. Indeed, knocking-down *MAPT* caused reduction of *C9orf72* whereas knocking-down *GRN* caused increased expression of *C9orf72*. Additionally, knocking-down *C9orf72* caused decrease *MAPT* expression and increased *GRN* expression (Rizzu et al., 2016).

Regardless, contribution of *C9orf72* loss-of-function to the pathology remains unclear. It is mostly accepted that *C9orf72* loss-of-function is not the main driver of disease onset. Results from cellular models points towards altered endosomal trafficking and autophagy pathways (Farg et al., 2014; O'Rourke et al., 2016) and in *C9ALS* cases, deregulation of genes implicated in the UPR pathway have also been observed (Prudencio et al., 2015). Animal studies allow a better understanding of *C9orf72* implications in disease pathology. Knocking-down *C9orf72* orthologues in zebra fish and *Caenorhabditis elegans* resulted in axonal and motility deficits (Ciura et al., 2013; Therrien et al., 2013). In another end, ablation of *C9orf72* in mice neurons did not cause any motor neuron dysfunction, altered survival, TDP43 mislocalization or astrogliosis or increased microglia activation (Koppers et al., 2015). Neither did treating adult-mice with *C9orf72* antisense oligonucleotides (ASOs) (Lagier-Tourenne et al., 2013). In the model described by Lagier-Tourenne and colleagues, intracerebroventricularly injected ASOs throughout the CNS of adult mice to induce depletion did not cause motor or cognitive dysfunction. Animals were tested from one month up to 4 months after injection. *C9orf72* RNA levels were decreased to 30% of normal expression 3 weeks after treatment, but this decrease was very transient and the expression of *C9orf72* was back to average levels 4 months after the initial injection. TDP-43 remained nuclear in cortex, hippocampus and cerebellum and no p62 or ubiquitin positive inclusions were observed. Germline knockout mice develop drastic hematopoietic abnormalities, splenomegaly, lymphadenopathy and premature death (Atanasio et al., 2016; Jiang et al., 2016; O'Rourke et al., 2016; Sudria-Lopez et al., 2016) suggesting major alterations in the inflammatory

response. Mild-motor deficits and body weight lost were observed in null mice appearing around 40 weeks without motor neuron loss or EMG abnormalities (Atanasio et al., 2016; Jiang et al., 2016). When explored, null mice also presented social recognition deficits starting at the age of 6 months (Jiang et al., 2016). Complete ablation of C9orf72 in mice presented a subset of lupus-like symptoms, namely lymphoid activation and hyperplasia with a characteristic autoantibody profile (Atanasio et al., 2016; Kurts et al., 2013). Additionally, a recent study showed alterations in gene expression of both sporadic ALS cases and C9ALS cases, predominantly in genes involved in the inflammatory response (Prudencio et al., 2015), further involving inflammation in C9orf72 pathology.

Even though implications of C9orf72 loss-of-function seemed limited through the study of these models, models using mutated HRE C9orf72 (BAC) also showed contradicting results (Jiang et al., 2016; Liu et al., 2016, 2016; O'Rourke et al., 2015).

### **2.3. Implications of the C9orf72 repeat expansion in cellular pathways: what do we know so far?**

Research in the past 5 years has been quite intensive in the C9orf72 field. Several mechanisms have been suggested implicating multiple cellular processes. Most of these processes are summarized in **Table 4**. A more detailed presentation of nucleolar stress, splicing alterations and nucleocytoplasmic shuttling are presented in the following section.

**Table 4** Pathological mechanism of the C9orf72 expansion *Adapted from Haeusler et al. (2016)*

C9orf72 HRE-linked cellular processes	Phenotype	Altered mechanisms	Associated genes, markers, proteins	Literature
<b>Nucleocytoplasmic trafficking</b>	Colocalization of RNA foci and DPRs with NPC components. Disrupted nuclear/cytoplasmic trafficking	RNA toxicity DPRs pathology C9orf72 loss-of-function	Importin $\beta$ , Ran-GTP, RanGAP, AlyRef	Zhang et al. (2015) Freibaum et al. (2015) Woerner et al. (2016) Xiao et al. (2015) Kwon et al. (2014)
<b>Stress granule dynamics</b>	Altered formation. DPRs colocalization with stress granules	RNA toxicity DPRs pathology	Purl-alpha, TDP-43	Tao et al. (2015) Wen et al. (2014) Farg et al. (2014) Rossi et al. (2015)
<b>P body dynamics</b>	Altered P body numbers	Unknown	DCP1	Wen et al. (2014) Haeusler et al. (2014)
<b>Axon excitability</b>	Hyper- or hypo-excitability	Unknown		Sareen et al. (2013) Geevasinga et al. (2015)
<b>RNA processing and maturation</b>	Increased alternative splicing. RNA nuclear retention	RNA toxicity DPRs pathology	hnRNP H, AlyRef, ADARB2, TDP43, Purl-alpha, RBPs	Kwon et al. (2014) Lee et al. (2013) Cooper-Knock et al. (2015) Prudencio et al. (2015)
<b>miRNA processing</b>	Dicer and Drosha colocalize with DPRs	DPRs pathology	Drosha	Porta et al. (2015)
<b>Nucleolar function</b>	Nucleolar perturbation. Altered ribosomal RNA biogenesis. DPRs colocalization with nucleoli	RNA toxicity DPRs pathology C9orf72 loss-of-function	NCL, FBL, B23 and ribosomal biogenesis	Kwon et al. (2014) Wen et al. (2014) Haeusler et al. (2014) O'Rourke et al. (2015) Schuldi et al. (2015)
<b>Autophagy</b>	P62 inclusions. Altered LC3II/LC3I levels. C9orf72 colocalization with vesicles	RNA toxicity DPRs pathology C9orf72 loss-of-function	C9orf72, LC3II, LC3I, UBQLN2, p62, Rab1, Rab11	Farg et al. (2014) Almeida et al. (2013) Zhang et al. (2012)
<b>Endoplasmic reticulum function</b>	ER stress sensitivity	RNA toxicity DPRs pathology	PERK-CHOP, IRE-XBP1	Zhang et al. (2014)
<b>Mislocalization of C9orf72 protein</b>	Altered C9orf72 localization to plasma membrane	C9orf72 loss-of-function	Rab7, Rab11, Ran-GTP, Importin $\beta$	Xiao et al. (2015) Farg et al. (2014)
<b>Endosome trafficking</b>	C9orf72 colocalization with endosomal proteins	C9orf72 loss-of-function	Rab7, Rab11	Farg et al. (2014)
<b>Neurite outgrowth</b>	Reduced dendritic branching	DPRs pathology C9orf72 loss-of-function	Unknown	Zhang et al. (2014) Ciura et al. (2013)

### 2.3.1. C9orf72 repeat expansion causes nucleolar stress

Nucleolin, an essential nucleolar protein, specifically binds the C9orf72 HRE in a highly robust manner (Haeusler et al., 2014). To explore the consequences of this binding, Haeusler and colleagues looked into nucleolin protein localization in C9orf72 patient lymphocytes and iPSC-derived motor neurons, and found abnormally dispersed staining in both cellular types. Nucleolin staining was condensed in the nucleolus in control cases and in non-C9ALS cases, and nuclear dispersion was observed in an expanded C9orf72 BAC mouse model, although downstream impairment was not observed (O'Rourke et al., 2015). Presence of nucleolin co-staining with RNA foci in patient motor cortex was also observed, supporting their interaction *in vivo*. Mislocalization of nucleophosmin, another nucleolar component, and decreased ribosomal RNA maturation further implicates nucleolar stress in the pathomechanism caused by the C9orf72 HRE (Haeusler et al., 2014).

Concomitantly, poly(GR) and poly(PR) colocalize to the nucleolus in *in vitro* models at micro molar concentrations (Kwon et al., 2014; Tao et al., 2015; Wen et al., 2014). Colocalization of these DPRs has also been observed with components of RNA granules (Yamakawa et al., 2014), which are important factors of TDP43 (and FUS) aggregate formation (Li et al., 2013). Of notice, altered nucleolar function has been observed in patients and models having decreased rRNA expression (Kwon et al., 2014), enlargement of nucleolus (Wen et al., 2014) and swelling with eventual fragmentation (Tao et al., 2015). Conversely, cytoplasmic localization of poly(GR) and poly(PR) have been described in a *Drosophila* model (Yang et al., 2015) and absence of nucleolar staining was described in patient spinal cord samples (Mackenzie et al., 2015; Schludi et al., 2015). Altogether, nucleolar stress seems to be caused by HRE products as both expanded RNA and DPRs seem to cause it.

### 2.3.2. C9orf72 repeat expansion causes splicing defects

Increased alternative splicing and alternative poly-adenylation was recently identified in c9ALS cases (Prudencio et al., 2015). Sequestration of RBPs and TDP43 mislocalization suggest altered function in RNA processing and transcription defects. Thus, several groups have looked into transcriptional profiles of c9ALS iPSC-derived neurons, fibroblasts, lymphoblastic cells, motor neurons, frontal cortex and cerebellum (Cooper-Knock et al., 2015a; Donnelly et al., 2013; Lagier-Tourenne et al., 2013; Prudencio et al., 2015; Sareen et al., 2013) and identified distinct transcriptome profiles specifically for c9ALS cases different from control and other ALS cases. These observations agree with results from RNA

sequencing clustering analysis of frontal and occipital cortices of C9orf72 patients (Anquetil et al. *in preparation*). In this data set, C9orf72 mutation carriers have ~10 times more defects in splicing than alterations in gene expression level, with a bias towards inflammatory/immune response and RNA processing. Altogether, RNA metabolism seems to be particularly impacted in C9orf72 pathology, but the causes leading these alterations remain to be identified.

### **2.3.3. C9orf72 repeat expansion causes nucleocytoplasmic transport impairment**

Compelling evidence in yeast and drosophila models has brought disruption of nucleocytoplasmic transport to the front of the debate about mechanism leading to C9orf72 pathology. Through different genetic screening methods to identify modifiers of C9orf72 HRE toxicity, several research groups identified components of nucleocytoplasmic shuttling as capable of modifying toxicity. Furthermore, they showed that C9orf72 expanded RNA and/or DPRs can alter expression of genes involved in nucleocytoplasmic transport or in conformation of the nuclear pore complex (Boeynaems et al., 2016; Freibaum et al., 2015; Jovičić et al., 2015; Zhang et al., 2015)., Although a common cellular pathway has been identified in all four studies, comparison of identified genes shows little overlap between the studies.

The expression of  $(G_4C_2)_{30}$  in *Drosophila melanogaster* lead to a neurodegenerative “rough eye” phenotype. Overexpression of RanGAP suppressed toxicity, whereas its knockdown caused increased HRE toxicity (Boeynaems et al., 2016; Zhang et al., 2015). Furthermore, TDP43 (TBPH in the fly) was found to have a higher cytoplasmic-to-nuclear ratio when the HRE was expressed. This observation suggests a mechanism that can trap TDP43 and cause its cytoplasmic mislocalization (Figure 27) (Zhang et al., 2015). No DPR pathology was identified in this model, making HRE solely responsible for the observed alterations. A similar model with 58 GGGGCC repeats presented DPR pathology, poly(GP) and poly(GR), but not poly(GA) (Freibaum et al., 2015). Flies with defined chromosome deletions were crossed with flies expressing the expansion, and the eye phenotype was analyzed. From this, 18 genetic modifiers were identified, all of which encode components of the nuclear pore complex or nuclear import/export proteins. DPRs toxicity was also observed in a yeast models expressing 50 copies of individual dipeptides without repetitive RNA species resulted in poly(PR) toxicity (Jovičić et al., 2015). Screening of genes modifying DPRs toxicity identified 78 genetic modifiers including multiple members of the karyopherin family, implicated in nuclear pore conformation.

In c9-iPSC-neurons, RanGAP preferential binding to C9orf72 sense RNA G-quadruplex was observed and decreased in protein import was shown by live imaging. As proteins larger than 40kDa require active transport to cross the NPC (Freitas and Cunha, 2009), both effects could lead to a RanGAP loss-of-function that will fail to activate nuclear protein import (Zhang et al., 2015) (Figure 27). Equally, Ran GAP1 knockdown acted as an enhancer of PR toxicity causing dysfunction in nucleocytoplasmic shuttling (Boeynaems et al., 2016). Furthermore, quantification of nuclear-to-cytoplasmic ratio of RanGAP and RCC1 protein showed abnormal redistribution to the cytoplasm (Jovičić et al., 2015). And in a recent mouse model it was shown that poly(GA) aggregates are capable of causing RanGAP and Pom121 mislocalization (Zhang et al., 2016). Inhibition of nuclear RNA export was also observed in HRE-expressing cells that presented irregularities in nuclear envelope architecture (Freibaum et al., 2015).

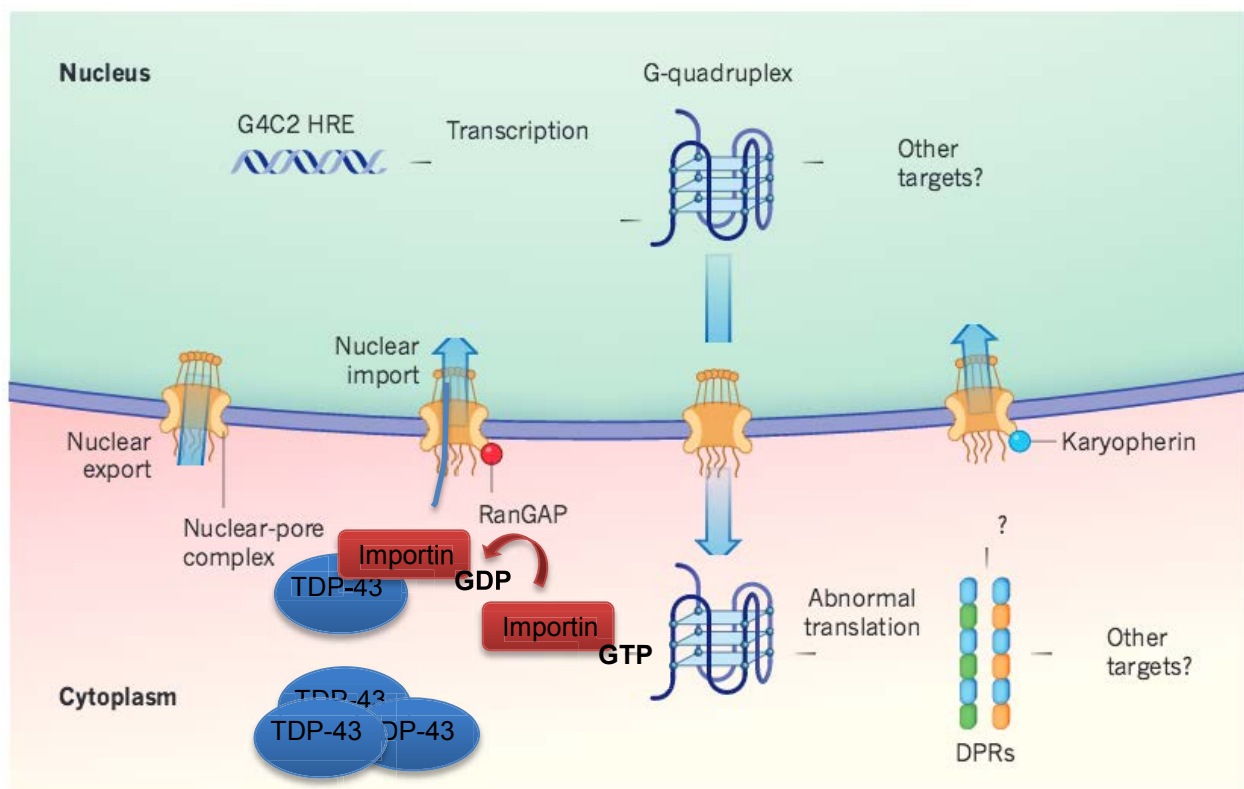
GLE1, an mRNA exporter protein, was identified as a genetic modifier of C9orf72 HRE toxicity (Freibaum et al., 2015). GLE1 is capable of interaction with nucleoporins (Folkmann et al., 2013), and regulates mRNA translation (Bolger et al., 2008). Homozygous GLE1 mutations cause prenatal motor neuron disease (Nousiainen et al., 2008) and heterozygous mutations are associated with ALS (Kaneb et al., 2015). Exportin 1 (Crm1 in flies) was also identified as a toxicity modifier. But contradictory effects have been described, where knockdown causes enhanced (Boeynaems et al., 2016; Freibaum et al., 2015) or suppressed (Zhang et al., 2015) rough eye phenotype. Exportin 1 is a karyopherin protein that is a critical receptor for protein export and necessary for the transport of RNAs across the nuclear envelope (Stade et al., 1997). Further studies are needed to determine implication of these modifiers in pathology.

Finally, given the importance of arginine methylases in nucleocytoplasmic shuttling regulation, modifiers were looked for in fly models (Boeynaems et al., 2016). Genetic screening identified several methyltransferases, including PRMT1 (Art1 in flies), as enhancers of toxicity suggesting modifications in epigenetic gene modulation.

Cytoplasmic aggregation of TDP43 (and FUS) observed in c9FTD/ALS cases could also be related to a dysfunction in nucleocytoplasmic shuttling. TDP43 normally shuttles between the nucleus and the cytoplasm to achieve its functions (Lagier-Tourenne et al., 2013). Mechanisms driving TDP43 nucleocytoplasmic shuttling remain poorly understood. Observations in normal cells have shown that the nuclear pore becomes leaky with aging (D'Angelo et al., 2009) and importins serving as cargo through the pore are down-regulated (Lu et al., 2004; Mertens et al., 2015). In pathological cases, aging could act as an aggravator

overwhelming the system and triggering cellular death. This could be the case in c9FTD/ALS. Sense and antisense RNA foci colocalize with the mRNA export factor AlyRef in motor neurons. If sequestered, AlyRef will not be able to export mRNA from the nucleus causing cellular imbalance (Cooper-Knock et al., 2015b, 2014). Simultaneously, cytoplasmic-aggregates, but not nuclear aggregates, will cause mislocalization of NPC components adding to defects in nucleocytoplasmic transport (Woerner et al., 2015).

Effects of the C9orf72 loss-of-function have not been explored in the context of nucleocytoplasmic shuttling, but a possible effect has been considered since the short isoform of the protein localizes to the nuclear envelope where it interacts with importin subunit  $\beta 1$  and



**Figure 27** Models of nucleocytoplasmic transport impairment mediated by GGGGCC toxicity

Transcription of these GGGGCC hexanucleotide repeat expansions (HRE) produces RNA transcripts that form G-quadruplex structures. These structures can be sequestered inside the nucleus, blocking and disrupting nucleocytoplasmic transport by binding to nuclear pore complex proteins. When they are exported to cytoplasm, G-quadruplex structures can either bind Ran GAP1 protein and inhibit the import process (and import of associated proteins, like importin linked to TDP43), or by undergoing abnormal RAN translation into dipeptide proteins that interfere with nuclear import, perhaps through interactions with karyopherin proteins (like importin). Of interest, the short isoform of normal C9orf72 protein localizes to the nuclear envelope where it interacts with importin subunit  $\beta 1$  and Ran GTPase. Adapted from Fox & Tibbetts (2015) and Zhang et al (2016)

RanGTPase. Interestingly, with age, members of the importin  $\beta$  family are down regulated and relocalized to cytoplasm in the frontal cortex and cerebellum (Mertens et al., 2015), as is the case for ALS spinal motor neurons (Xiao et al., 2015), so a combined effect could take place. Altogether, these data suggest a combined effect of HRE expansion mechanisms (RNA foci, DPRs and loss-of-function) affecting a common cellular process, which is nucleocytoplasmic shuttling. The extent to which every mechanism will independently affects the shuttling remains yet to be studied.



## V. Aims of the study and choice of model

Although available models have provided great insight into underlying mechanisms of c9FTD/ALS, several questions remain yet unanswered. One of the main questions concerns the role of the loss-of-function in pathogenesis and degeneration, as we still do not know what are the upstream cellular events and triggers that will cause neuronal death. As both FTD and ALS present TDP43 inclusions, we still wonder what events will lead to TDP43 aggregation and to its nuclear depletion? Is the mislocalization of TDP43 a cause or rather a consequence of ALS and/or FTD? And what are the links between the C9orf72 HRE lead and TDP43 protein mislocalization?

Understanding underlying mechanisms will allow the development of new treatments targeting specific pathways that lead to neurodegeneration, but also to develop specialized biomarkers that will be useful in diagnosis and patient care. Mouse models are a useful tool for deciphering altered cellular processes at different stages of the disease and allow for testing of both treatments and biomarkers in a physiological system. With the insight from gain-of-function models, several targets have already been identified and ASOs treatment has been proposed to diminish observed toxicities from the products of the HRE, the expanded RNA and the DPRs. However, as existing mouse models do not always mimic neuronal death, TDP43 pathology or altered cognitive phenotype, caution should be taken before administering drugs or other treatments to patients, as side effects could develop. Down regulation of protein levels could result in important immunological alterations or other yet unidentified effects, thus further characterization of underlying disease mechanisms needs yet to be acquired.

The main aim of this thesis was to develop and characterize a new mouse model deficient in C9orf72. The goal was to obtain a valid C9orf72 loss of function model that can be further used to identify cellular abnormalities or imbalance caused specifically by this particular alteration. For this, we have generated a ubiquitous C9orf72 deficient mouse model using a miR-RNAi to target and down regulate the murine C9orf72 protein. By using lentiviral transgenesis, we can obtain a slightly variable expression of the final product of *c9orf72* mouse orthologue to mimic observed variations in patients cohorts (Ciura et al., 2013) and possibly obtain expression-related phenotypes. During my PhD, I extensively characterized the behavior of transgenic miR-C9orf72 animals in tests related to both FTD and ALS

(**Figure 28**) to look up for anomalies relating to either, or both of these diseases. In parallel of the behavioral characterization, I also did an extensive histopathological exploration of brain and spinal cord of C9orf72 deficient animals to identify hallmarks of FTD and ALS pathology, particularly TDP43 and p62 aggregation, but also neuronal and/or motor neuronal loss. With this study, we hope to determine if the loss-of-function has a role in driving FTD or ALS pathology and if the mice developed could constitute a good model for understanding this aspect of the disease.



**Figure 28** Behavioral characterization of miR-C9orf72 mice

In the following section I will present the results of this extensive characterization and in the final part I will discuss the implication of C9orf72 loss-of-function in FTD and ALS pathology according to results obtained from the characterization.

# **Results: Partial C9ORF72 loss of function in mice leads to mild behavior anomalies evoking FTD-like symptoms without motor neuron disease (Lopez-Herdoiza et. al)**

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**SUMMARY**

The GGGGCC intronic repeat expansion within *C9ORF72* is the most common genetic cause of ALS and FTD. The mutation may cause a toxic gain of function either through accumulation of RNA foci that capture RNA-binding proteins; or through aggregation of dipeptide repeat proteins produced by RAN translation. A partial decrease of *C9ORF72* transcripts and protein level is also observed in patients, implying a concomitant loss of function mechanism. We have generated *C9ORF72* knock-down mice to investigate the role of this loss of function in the pathogenesis. These mice develop deficits in social interaction and a depression like behavior, both anomalies related to FTD, but no motor neuron disease apart from a late strength loss and neuromuscular junction deficits. These findings suggest that *C9ORF72* partial loss of function leads disease course more towards FTD while RNA foci and/or C9RAN proteins may be needed to trigger motor neuron disease and massive neurodegeneration.

## INTRODUCTION

Hexanucleotide repeat expansion (HRE) located in the 5' UTR region of *C9ORF72* gene is the most common genetic cause of familial frontotemporal dementia (FTD) and amyotrophic lateral sclerosis (ALS) (DeJesus-Hernandez et al., 2011; Gijssels et al., 2012; Renton et al., 2011). These two fatal neurodegenerative diseases have been known to occur within the same families, suggesting a phenotypical continuum (Burrell et al., 2016; Vance, 2006). Clinical symptoms of motor neuron disease have been seen in over 30% of FTD patients, and up to 50% of ALS patients develop impairment in cognition and behavior (Giordana et al., 2011; Lomen-Hoerth et al., 2002). The precise mechanism or mechanisms through which the *C9ORF72* mutation causes “c9FTD/ALS” has not yet been elucidated, but several hypotheses have been proposed. The mutation may cause a toxic gain of function either through transcription of sense and antisense expanded RNA that aggregate and capture RNA-binding proteins impeding them to have their normal function and thus, eventually leading to cell death (Donnelly et al., 2013; Lee et al., 2013; Loureiro et al., 2016; Sareen et al., 2013); or through the translation of aggregating peptides that are produced by repeat-associated non-AUG (RAN) translation (Ash et al., 2013; Mori et al., 2013). These dipeptide repeat proteins (DPRs) or “c9RAN proteins” cause degeneration in cell culture, drosophila models and mice models (Ash et al., 2013; Mizielinska et al., 2014; Mori et al., 2013; Zhang et al., 2016). Both the RNA foci and the c9RAN proteins are present in the brains of patients carrying the *C9ORF72* mutation (DeJesus-Hernandez et al., 2011; Gendron et al., 2015; Gomez-Deza et al., 2015; Mackenzie et al., 2015). Furthermore, the loss of one to three of alternatively spliced *C9ORF72* transcripts and a decreased level of C9ORF72 protein have been observed in the frontal cortex of patients implying a concomitant loss of function mechanism (Belzil et al., 2013; DeJesus-Hernandez et al., 2011; van der Zee et al., 2013; Waite et al., 2014). Neurodegeneration can then occur through multiple pathogenesis mechanisms. As a matter of

fact, both gain and loss of function models present contradicting results that shun interpretation of implicated physiological pathways in disease. Several gain of function models reproduce c9ALS/FTD histopathological lesions without (O'Rourke et al., 2015; Peters et al., 2015) or with mild (Jiang et al., 2016; Liu et al., 2016) associated behavioral modifications while others present both the histological hallmarks and the behavioral deficits when overexpressing the HRE (Chew et al., 2015; Zhang et al., 2016). On the other hand, the function of *C9ORF72* remains largely unknown, but it has been suggested to play a role in protein trafficking and more precisely in nucleocytoplasmic shuttling and/or endosomal trafficking (Farg et al., 2014; Freibaum et al., 2015; Levine et al., 2013; Loureiro et al., 2016; Xiao et al., 2015). Complete *C9ORF72* depletion in mice causes abnormal peripheral immunity and abnormal social recognition with minor motor deficits at 12 months of age (Atanasio et al., 2016; Jiang et al., 2016; O'Rourke et al., 2016). Conversely, intraventricular injection of antisense-oligonucleotide targeting *C9ORF72* in CNS did not produce motor deficits 4 months after treatment (Lagier-Tourenne et al., 2013). In the same line, heterozygous depletion of *C9ORF72* or neural-specific conditional knock-out mice did not develop motor neuron degeneration, defects in motor function or altered survival (Atanasio et al., 2016; Jiang et al., 2016; Koppers et al., 2015; O'Rourke et al., 2016). These results suggest that *C9ORF72* deficiency is not the sole or more potent trigger of neurodegeneration in c9ALS/FTD, but its exact contribution to the phenotype has not yet been fully understood. Comprehensive analysis and comparison of several independent loss-of-function mice models, as diverse as possible, is critical to determine the effect of *C9ORF72* deficiency and its role in the pathology. Importantly, the exact loss of function effect of the mutation is a down-regulation of 20% to 70% of the expression of the *C9ORF72* transcripts (Ciura et al., 2013; DeJesus-Hernandez et al., 2011; Gijssels et al., 2012; Haeusler et al., 2014; Renton et al., 2011; van der Zee et al., 2013), which might be better mimicked by a knock-down than by

a heterozygous knock-out of the gene. Despite advances made in the understanding of c9FTD/ALS pathogenesis, further understanding of how these mechanisms contribute to disease triggering and evolution is needed.

In order to understand the possible contribution of the partial loss of function mechanism to the disease, we have generated a ubiquitous knock-down mouse model by targeting the mouse RNA homologue of *C9ORF72*. Extensive behavioral and histological characterization was undertaken to determine if mice develop phenotypical and pathological features of c9FTD/ALS. We found that by knocking-down *C9ORF72* intrinsically, mice develop cognitive deficits in social interaction and depression like behavior, which relate to FTD-like anomalies. . When investigating ALS-like abnormalities, we found that miR-*C9ORF72* mice have a normal motility throughout their lifespan but present a lessening of strength that appears at an advanced age (12 months) and is maintained without further aggravation. Those mice do not present neuronal or axonal loss or signs of muscle atrophy and/or wasting, and synaptic transmission appears preserved. However, we detected subtle abnormalities at the neuromuscular junction (NMJ), including signs of very mild distal axonal suffering, that aggravate with aging. Finally, *C9ORF72* deficient mice did not present increased glial activation or significant TDP43 mislocalization when compared to controls, but presented increased p62 protein accumulation.

These findings suggest that *C9ORF72* partial loss-of-function could cause cellular fragility driving disease to a more FTD-like course in the mouse. RNA foci, c9RAN proteins or both are probably needed to trigger neurodegeneration in both brains and spinal cords.

## RESULTS

### **Depletion of *C9ORF72* does not cause altered survival or weight loss**

We have designed a miR-RNAi targeting a region located in exon 8 of the *c9orf72* mouse orthologue, which is common to all transcript variants, to knock-down the expression of

C9ORF72 (Figure 1A), and thus mimic the down-regulation effect seen with the human mutation. A lentiviral vector containing this miR-RNAi sequence and a GFP reporter gene under the control of a ubiquitous promoter (phosphoglycerate kinase, PGK, Figure 1A) was injected in mouse oocytes. Founder mice were then selected to create a lineage of transgenic mice with a range of knock-down of C9ORF72 (miR-C9ORF72), as was observed in patients cohorts (Ciura et al., 2013; DeJesus-Hernandez et al., 2011). Similarly, transgenic mice expressing a scramble miR-RNAi were generated as control animals (miR-Scramble, scr). An initial verification was performed at E14.5 confirming lentiviral expression ubiquitously in transgenic mice (Figure 1A). C9ORF72 mRNA quantification was then performed at 2 and 9 months of age (not shown) and at 23 months of age in the experimental group that underwent behavioral and histological characterization (Figure 1 B). The relative C9ORF72 mRNA expression in this group is stably decreased at all ages tested and in all regions of interest. It is down to 55% in C9ORF72 deficient animals when compared to wild-type mice or scramble controls in the cortical region (vs. wild type (wt) or scr  $p < 0.0001$ ) and in spinal cord (vs. wt  $p = 0.0003$  and scr  $p < 0.0001$ ), but also in the muscle (vs. wt  $p = 0.001$ , vs. scr  $p = 0.017$ ) (Figure 1B). In addition, western blotting with a specific C9ORF72 antibody showed a ~60% decreased level of protein expression ( $p = 0.0026$ , t-test) in the cortex of C9ORF72 deficient mice (Figure 1C). Taken together, these results demonstrate a ubiquitous knock-down of C9ORF72 of approximately 45% (RNA levels) and 60% (protein levels), making the model correlative to levels of expression seen in patients (Ciura et al., 2013; DeJesus-Hernandez et al., 2011).

C9ORF72 deficient mice were equal to controls in viability, appearance, fertility and weight (Figure 1E). Finally, survival was not significantly affected in these animals, as they were still alive after 23 months of age (Figure 1F).



**C9ORF72 deficient mice have altered social interaction and depression like behavior**

In order to identify whether decreased expression of C9ORF72 caused alterations of social interaction, anxiety-like or depression-like behaviors, as was the case in other FTD models (Filiano et al., 2013; Roberson, 2012; Yin et al., 2010), miR-C9ORF72 mice were subjected to a battery of behavioral tasks at 2, 5, 9, 12, 15 and 18 months of age.

Social withdrawal is a common symptom of FTD (Burrell et al., 2016). Low social approach in mice represents a phenotype reminiscent of social deficits observed in FTD patients that can be investigated using the three-chamber social interaction test (Yang et al., 2011). Mice are social animals that will have a natural tendency to approach and investigate unknown individuals by sniffing. When compared to controls, C9ORF72 deficient mice were unable to distinguish between the “novel mouse” and the “known mouse” (50% vs. 50% of total sniffing time), from 5 months of age onward through all subsequently tested ages (Figure 2A). Thus, miR-C9ORF72 mice present deficits in social interaction. Of note, when confronted with the first probe mouse or an unanimated object, all mice preferred the mouse rather than the object (Figure S1A). Furthermore, we verified olfaction abilities with the habituation/dishabituation test, showing that miR-C9ORF72 mice are perfectly able to identify a new odor and to differentiate between a social odor and a non-social one (Figure S1B). These results show that C9ORF72 deficiency alters social recognition specifically as early as 5 months of age.

In early stages of FTD pathology, patients present a loss of initiative and neglect of personal hygiene; behaviors that resemble symptoms of depression. The forced swim test has been classically used to measure depression-like behavior in mice (Porsolt, 2000). In this test, C9ORF72 deficient animals presented an increased depression like behavior: miR-C9ORF72 mice present longer periods of immobility at 5, 9, 12, 15 and 18 months of age when compared to controls (Figure 2B). When accounting for exploration or anxiety-like behavior

in a novel-environment with the open-field test and the dark and light chamber test, no difference was observed among groups; neither was an impairment of spatial memory observed in the Morris-water maze test (Figure S1C-D).

In parallel, we evaluated whether decreased expression of C9ORF72 caused neuronal loss or degeneration in the brain. At 23 months of age, miR-C9ORF72 mice did not present brain atrophy or a marked reduction of whole cortex area (Figures 1C-D and S1E-F). The number of NeuN-positive neurons in frontal cortex and motor cortex was similar in C9ORF72 deficient mice when compared to controls (Figure 2E-F). These results suggest that C9ORF72 deficiency causes cognitive anomalies in mice but does not directly affect neuronal survival.

### **Synaptic transmission is not altered in hippocampal networks of C9ORF72 deficient mice**

A recent study demonstrated the involvement of the hippocampus area in the social interaction behaviors (Hitti and Siegelbaum, 2014). Since miR-C9orf72 animals displayed some similar cognitive deficits without signs of neurodegeneration (Figure S1G), we investigated the neuronal physiological functions in the hippocampus and in particular the synaptic transmission properties. Electrophysiological responses at the Schaffer collateral-CA1 synapse were recorded in acute hippocampal slices of C9orf72 deficient (n=7), miR-scramble (n=7) and wild-type animals at 18 months of age (Figure 3). No modification in the basal synaptic transmission was detected between the miR-C9ORF72 animals and the controls. The amplitude and the slope of the CA1 hippocampal field excitatory post-synaptic potentials (fEPSPs) exhibited normal and similar responses to increasing stimulation intensities of the CA3 Shaffer collaterals (t-test,  $p=0.13$  for the fPSP slope and  $p=0.18$  for the fPSP amplitude; Figure 3B). We explored the synaptic plasticity mechanisms of the CA3 – CA1 pyramidal cells synapses. Studying the fEPSP paired-pulse ratio (EPSP2/EPSP1) of the wild-type animals showed that synapses tended to facilitate (paired-pulse facilitation, PPF)

after a delivery of 2 stimulations at a frequency of 0.02Hz. No change was detected in the short-term plasticity since the miR-C9ORF72 mice slices exhibited a similar PPF behavior in response to different stimulation intervals (t-test,  $p=0.24$ ; Figure 3C). Finally the long-term plasticity (LTP) was investigated by delivering a high frequency stimulation of the Shaffer collaterals (x2, 100Hz, 1s). In wild-type slices, the fEPSPs amplitude was potentiated to 125% at 30min from  $1 \pm 0,01$  to  $1,25 \pm 0,01$  (t-test;  $p<0.01$ ,  $n=6$ ). The LTP amplitude was similar in C9ORF72 deficient mice and scramble controls (t-test;  $p<0.01$ ,  $n=6$ ; from  $1 \pm 0,01$  to  $1,22 \pm 0,01$ ). These data show that the hippocampal synaptic transmission is not impaired in the C9ORF72 deficient animals.

### **C9ORF72 deficient mice show normal balance and motor activity and no motor neuron loss**

To test whether ALS-like anomalies arise when knocking-down C9ORF72, we assessed motor performance and motor neuron degeneration. With the accelerating rotarod C9ORF72 deficient mice spent the same time on the rotating rod through the different tested time points when compared to controls, implying normal balance and coordination (Figure 4A). To further assess miR-C9ORF72 mice locomotion, video footages of the three groups walking on a treadmill were analyzed in a frame-by-frame basis. Gait traces were produced from which step, stance and swing duration were analyzed. A regularity index was then computed. No major difference was observed in gait coordination and execution of step timing (Figure S2A). No difference in travelled distance or velocity was observed in the Open-Field (Figure 4B). Thus, no major locomotion deficits were observed when knocking-down C9ORF72.

We used electromyogram recordings (EMG) to determine whether neurophysiological abnormalities occur in the peripheral nervous system. Compound muscle activated potential (CMAP) amplitude as well as distal motor latency (DML) were normal, thus excluding major motor conduction changes in 20 month-old miR-C9ORF72 mice (Figure 4C).

Next, we evaluated whether knocking-down C9ORF72 could cause motor neuron degeneration. No difference in motor neurons numbers or morphology was observed between groups at 5-12 and 22 months of age (Fig 4D). Thus, C9ORF72 deficiency alone in mice causes neither locomotion deficits nor motor neuron loss.

### **C9ORF72 deficient mice present mild strength loss and neuromuscular junctions morphological abnormalities**

Mice were tested for their muscle strength using the grip test, where the peak of maximal force is measured for hind limbs. MiR-C9ORF72 mice were slower to reach their adult peak of force (inferior strength at 2 months compared to wt,  $p=0.009$ , and scr,  $p=0.009$ ) and then presented a deficiency that appeared at 12 months (vs. wt  $p=0.007$ , vs. scr  $p=0.009$ ) and lasted throughout life (Figure 5A). Similar results were observed with the hanging wire test confirming the observed effect (vs. wt  $p=0.03$ ; vs. scr  $p=0.021$ ; Figure S2B).

Reduced muscle strength may result from motor neuron loss, neuromuscular transmission failure, or muscle atrophy. As motor neuron loss was not observed we looked into other possible mechanisms. Neuromuscular junction innervation can be deeply affected in ALS (Dupuis and Loeffler, 2009), thus we looked for signs of degeneration and/or muscle atrophy. We tested neurophysiologically whether functional denervation or defective neuromuscular transmission took place in C9ORF72 deficient mice. Muscle fibrillations were most frequently observed in 20-month-old miR-C9ORF72 mice compared to miR-scramble mice ( $p=0.45$ ) (Figure 5B), which suggested events of muscle denervation. However, no significant decrement in the EMG at repetitive nerve stimulation pointing on neuromuscular transmission failure was observed whatever conditions of nerve stimulation were used, excluding these defects as being massive (data not shown).

We then studied individually the NMJs at the morphological level. We did not observe denervation events in the miR-C9ORF72 mice as suggested by the EMG data (Figure 5C).

However, we observed a mild yet significant remodeling of pre- and post-synaptic compartments (Figure 5D). Unlike wild-type and miR-Scramble mice in which we observed typical fork-shaped nerve terminals innervating well-defined postsynaptic pretzel organization of the acetylcholine receptors (AChR), the miR-C9ORF72 mice presented pre- and post-synaptic defects in all tested muscles at 15 and 23 months of age (Figure 5D). In the soleus muscle and diaphragm, fragmentation of post-synaptic gutters and focal pre-terminal axonal swellings were most frequently seen in miR-C9ORF72 mice when compared to controls (Figure 5D). Finally, muscle fibers did not present changes at the histological level (Figure S2C). Thus, C9ORF72 deficiency caused deficits in muscle strength that correlated with NMJ structural alterations without changes in neuromuscular transmission detectable in whole muscles.

#### **C9ORF72 deficient animals do not present reactive gliosis or TDP-43 inclusions but show accumulation of p62**

Astrocytes and microglia activation are implicated in the onset and progression of neurodegeneration in both ALS and FTD (Radford et al., 2015). To test whether C9ORF72 deficient animals had increased glial activation, we quantified the number of GFAP positive cells in the frontal and motor cortex at 12 and 23 months. Overall, there was no significant difference between miR-C9ORF72 mice and controls in the number of GFAP-positive cells (vs. wt, scr  $p=0,2$ ) (Figure 6B). We then looked for increased microglia activation in miR-C9ORF72 mice. For this, we stained brain slices with Iba-1, a common microglia marker. Positive Iba-1 cells were not more abundant in the frontal cortex of miR-C9ORF72 mice when compared to controls ( $p=0,4$ ) (Figure 6A).

Cytoplasmic inclusions of phosphorylated TDP-43 and p62 are a major hallmark of c9FTD/ALS pathology (Mackenzie et al., 2014; Neumann et al., 2006). In order to identify TDP-43 pathology, we quantified cytoplasmic staining of TDP-43 in both frontal and motor

cortex in 23 months old animals. Despite a relatively high percentage of cells with cytoplasmic TDP-43, C9ORF72 deficient mice did not present significantly more cytoplasmic TDP-43 when compared to controls, neither in frontal nor in motor cortex (Figure 6C). Regarding p62, C9ORF72 deficient animals presented twice more cells containing p62-positive structures when compared to controls (Figure 6D; vs. wt  $p=0.005$ ; vs. scr  $p=0.029$ ). Cells positive for p62 were more numerous in the frontal cortex (18%) than in the motor cortex (10%) (not shown). Furthermore, p62 staining colocalized with lysosomal marker Lamp1 in C9ORF72 deficient animals.

Taken together, these data show that C9ORF72 deficiency alone is not sufficient to trigger significant glial activation in the brain of mice, or to cause TDP-43 pathology but can lead to p62 accumulation thereby implying alterations of the lysosomal pathway.

## DISCUSSION

Mechanisms that drive neurodegeneration in C9ORF72 ALS/FTD have raised a number of questions, as models have failed to identify a main triggering effect. On the one hand, RNA foci and C9RAN proteins seem to cause neurodegeneration and often lead to motor neuron disease when overexpressed, although some models produce RNA foci and RAN proteins but do not develop neurodegeneration or behavioral phenotypes (O'Rourke et al., 2015; Peters et al., 2015). On the other hand, the intronic HRE can inhibit promotor activity (Liu et al., 2014; Russ et al., 2015; van der Zee et al., 2013; Xi et al., 2013) causing a decrease of C9ORF72 gene expression but the effect of this partial loss of function in regard to the pathology has not been fully studied yet. Actually, knock-out mice have been produced shedding light on the functions of the C9ORF72 protein in the immune system, but behavioral characteristics of these mice, in particular related to FTD, have not been extensively investigated (Atanasio et al., 2016; Burberry et al., 2016; O'Rourke et al., 2016; Sudria-Lopez et al., 2016) except in one case (Jiang et al., 2016). In that study, Jiang et al. showed that complete ablation of

C9ORF72 resulted in sociability defects and late motor deficits. Heterozygous mice did not present this phenotype but C9ALS/FTD patients express variable C9ORF72 mRNA levels, ranging from 20% to 70% of transcript decrease in cortex, with less decrease in other areas (Ciura et al., 2013; DeJesus-Hernandez et al., 2011; Gijssels et al., 2012). In our study, mice with a ubiquitous C9ORF72 knock-down develop several but mild pathological features of C9ALS/FTD. Our results show that the partial loss of function of C9ORF72 in mice causes cognitive anomalies by 5 months of age without developing major motor dysfunction. Additionally, these animals develop subclinical signs of impaired synaptic transmission events associated with axonal swelling at the neuromuscular junction (NMJ) at later stages. Together, these data demonstrate that decreased C9ORF72 is a contributor factor to pathogenesis in c9ALS/FTD. We conclude that the partial loss of function of C9ORF72 is important for the development of C9ALS/FTD pathology, although it is not enough to drive or trigger neurodegeneration.

During this study, we performed an extensive behavioral characterization of our model. The miR-C9ORF72 mice presented alterations in social interaction, as seen in the three-chamber test, but were perfectly capable of differentiating unanimated objects and various social stimuli (Figure S1A-B) as well as learning and memorizing a maze (Figure S1D). Their abnormal behavior is therefore selectively affecting social recognition and not just recognition or memory per se. Similarly, these mice developed a depression like behavior in the forced swim test that was not tainted by a motor phenotype or a global decrease in activity. Frontal and temporal lobe degeneration proper to FTD is characterized by behavioral changes such as disinhibition, apathy and inappropriate social behavior. Our findings are consistent with observed FTD behavioral deficits. Other FTD mouse models develop memory deficits with aging (Yin et al., 2010), but this feature is not commonly seen in patients with FTD-C9ORF72 and was not observed in miR-C9ORF72 mice. These behavioral changes were not

accompanied by neuronal loss, astrogliosis or increased activation of microglia. Major locomotor deficits, weight loss, survival alterations or muscular atrophy were not present in these animals. However, decreased strength in hind limbs coupled to feebleness when hanging to an inverted grid and pre and post-synaptic alterations in the NMJ denotes subclinical suffering of the motor unit. The observed focal axonal swellings in the miR-C9ORF72 mice might represent early ultra-structural signs of future and most pronounced axonal damages preceding axon morphology changes (Nikic I. et al., 2014; Duregotti E. et al., 2014). “Focal axonal degeneration” (FAD) are characterized by sequential stages, beginning with focal axonal swellings and progressing to axon fragmentation, eventually leading to motor neuron death (Craner and Fugger, 2011; Nikić et al., 2011).

In the first models of C9ORF72 loss of function in mice, animals did not develop either locomotion defects or motor neuron loss, nor showed subclinical signs of degeneration. C9ORF72 reduction was either exclusively neuronal (Koppers et al., 2015) or had only transient knock-down (Lagier-Tourenne et al., 2013). Conversely, ubiquitous knock-out mice showed social behavior incongruities by 6 months of age and presented motor deficits and decreased activity at 10 months of age but without appearance of motor neuron loss or EMG abnormalities (Atanasio et al., 2016; Jiang et al., 2016). The heterozygous mice in these studies did not develop any quantified anomalies (Jiang et al., 2016). The phenotypic profile of our miR-C9ORF72 mice is somehow intermediate, and helps to identify strong reproducible effects of C9ORF72 reduction, therefore supporting the interest of knock-down models. By comparing the models, it appears that decreasing C9ORF72 in the mouse results consistently in social interactions deficits and mild strength decrease while motor activity is unaffected.

Several groups showed recently that complete ablation of *C9ORF72* causes mice to develop an autoimmune response comparable to human systemic lupus erythematosus (SLE)



(Atanasio et al., 2016; Jiang et al., 2016; O'Rourke et al., 2016). This discovery provides insight into the possible roles of the *C9ORF72* protein. Heterozygote mice do not seem to suffer from this abnormal immune activation, although further studies are needed to know the real impact of *C9ORF72* hemi-dose on the immune system. This type of effect has been observed with the FTD-causing gene progranulin (*PGRN*). Homozygous *PGRN* mutations causes patients to develop neuronal ceroid lipofuscinoses (NCLs), a systemic auto-immune disease, whereas heterozygous mutations will rather cause behavioral FTD (Smith et al., 2012). *PGRN* knock-out mice presented increased inflammatory and phagocytic responses with changes in behavior (Lui et al., 2016; Yin et al., 2010); while heterozygous mice present FTD-related behavioral deficits without major inflammatory alterations (Filiano et al., 2013). Whether similar mechanisms take place with *C9ORF72* loss of function is yet to be elucidated. Preliminary results in heterozygous knock-out mice and our miR-*C9ORF72* mice suggest that, in these mice also, there is no major neuro-inflammation. However, there is a strong possibility that more subtle alterations of the immune response can generate a critical frailty and contribute significantly to the disease. It is interesting to note that both *C9ORF72* and *PGRN* depletion are capable of causing systemic immune dysfunction in mice models and thus further research concerning the immune response in FTD pathology will be of great interest. FTD-*PGRN* patients do not develop motor neuron disease and from our study and concomitant mice models, the ablation of *C9ORF72* does not cause motor dysfunction either. As c9ALS/FTD patients preserve 30 to 80% of *C9ORF72* transcript expression in the brain (Ciura et al., 2013; DeJesus-Hernandez et al., 2011; Gijselinck et al., 2012), the question regarding the minimal *C9ORF72* levels required to preserve immune functions remains yet to be elucidated, as whether age- and stress-related lesions will overload an already fragile system. Interestingly, *C9ORF72* has been shown to interact with several Rabs, including the endocytic Rab5 and Rab7, which are components of the autophagy response (Almeida and

Gao, 2016; Farg et al., 2014; Xiao et al., 2015). The p62 protein, which is aggregated in the brain of C9ORF72 expansion carriers, is also associated to autophagy. In our mice, we observed a significant increase in p62-protein staining in the cortex that colocalized with lysosomes. This result show that decreased expression of C9ORF72 might dysregulate the autophagic/lysosomal pathway, in agreement with previous results (O'Rourke et al., 2016; Sellier et al., 2016), and so contribute to or modulate disease pathogenesis.

Taken together, our study provides new insight into the understanding of c9ALS/FTD pathology, suggesting that decreased expression of C9ORF72 may specifically sensitize target neuronal cells to damaging stimuli or stress such as those caused by RNA foci or DPRs that may be necessary to trigger the full neurodegenerative c9ALS/FTD.

## **EXPERIMENTAL PROCEDURES**

Please see the **Supplemental Experimental Procedures** for more information

### **C9ORF72 miR-RNAi generation**

Generation of miR-RNAi anti-*c9orf72* (miR-C9ORF72) mice was done using a lentiviral vector carrying a ubiquitous PGK promoter driving the expression of the 5'-TTGACATCCACATCAATGTGCGTTTTGGCCACTGACTGACGCACATTGGTGGATGTCAA-3' sequence targeting mouse *c9orf72* transcript variants 1, 2 and 3, coupled to the expression of EmGFP. It was injected in C57Bl6/N mice oocytes and a similar vector carrying a random miR-RNAi sequence was used for scramble controls (miR-Scramble).

All animals experiments were approved by the institutional animal care and use committee N°2010/63 UE European legislation and national authority (Ministère de l'Agriculture, France) guidelines.

**C9ORF72 knock-down validation**

C9ORF72 mRNA quantified by qPCR in cortex, spinal cord and muscle. Primers and probe sequences are listed in Table S1. C9ORF72 expression was analyzed by immunoblot of whole brain RIPA lysates probed with homemade rat monoclonal C9ORF72 antibody. Quantification of C9ORF72 signal intensities normalized to GAPDH was done using the analysis software AIDA (Raytest GmbH).

**Histological analysis**

Sections of paraffin-embedded brain and spinal cord were stained using standard protocols with antibodies against Iba1, GFAP, Neun, TDP 43, p62. Digital images were captured with slide scanner Axioscan (Zeiss) or with Apotome (Zeiss) at 1,5 and 2,0mm from interneural line. Whole mounts specimens of soleus, extensor digitorum longus (EDL) and diaphragm muscles were stained for AchR with TRITC-labeled  $\alpha$ -bungarotoxin, for hNL168, SV2 and images were acquired with a confocal microscope (Olympus FV-1000). Neuronal loss quantification was done by counting the totality of neurons in frontal and motor cortex using Stereo Investigator software (MBF Bioscience). Cell counts for positive Iba1 and GFAP cells was done on images covering frontal and motor cortex. Positive staining for cytoplasmic TDP 43 and p62 was counted in a total of 200 to 250 cells; positive cells for cytoplasmic staining were normalized to total number of TDP-43 positive cells. Lumbar motor neurons marked with Nissl staining were quantified in Leica DM250 microscope (20x). Morphological endplate count was done on confocal images. All cells counts were done by an experimenter who was blind to genotype and counts were reported to the surface area ( $\text{mm}^2$ ) using ImageJ software (National Institutes of Health).

## **AUTHORS CONTRIBUTIONS**

M.-B.L.-H., C.L-D., S.N., M.L. designed experiments and analyzed data. S.B., B.W., D.R., J.B., P.F., P.G., C.D., performed the experiments and analyzed data. M.F., D.B., S.D., performed the experiments. M.D., M.N., G.B., P.R. contributed methodology. I.LB. and A.B helped design and coordinate the project. M.L. planned, designed and coordinated the project. M.-B.L.-H. and M.L. wrote the manuscript.

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**FIGURE LEGENDS****Figure 1: Generation of C9ORF72 deficient mice**

- (A) Schematic representation of C9ORF72 transcripts in the mouse and the lentiviral vector used to perform transgenesis. MiR-C9ORF72 targeted region is denoted by a square zone. Ubiquitous transgene expression (visualized by GFP fluorescence) shown in mouse embryo (right).
- (B) Relative expression of C9ORF72 in transgenic mice cortex, spinal cord and muscle measured by qPCR.
- (C) Levels of mouse C9ORF72 ortholog in brain tissue of miR-C9ORF72 and wild-type mice. The expression of C9ORF72 was quantified by densitometric analysis of western blots and normalized to GAPDH. Note that only isoform 3 (~50kDa) is detected at the protein level in the mouse brain. The positions of the molecular weight marker are indicated on the left in kDa.
- (D) Weight curve of male (left) and female (right) miR-C9ORF72 mice compared to controls (wt n=10; scr n=12, c9 n=34).
- (E) Survival curve up to 24 months (wt n=10; scr n=12, c9 n=34)
- Error bars represent SEM; n.s. non-significant; \*p<0.05, \*\*p<0.01.

**Figure 2: C9ORF72 deficient mice develop behavioral abnormalities**

- (A) Three-chamber test for sociability and social novelty at 2-5-12 and 18 months (wt n=10; scr n=12, c9 n=34). Social exploration is quantified as time spent sniffing an already known mouse (stg1) or a novel mouse (stg2).
- (B) Forced swim assessment for depression-like behavior at 2-5-12 and 18 months (wt n=10; scr n=12, c9 n=34). Depression-like behavior is measured as the time of immobility in the water tank.
- (C) Areas of the cortex analyzed: total cortex (yellow), frontal cortex (green) and motor cortex (purple). Scale bar= 1000µm
- (D) Quantification of the total cortical surface.
- (E) Immunofluorescence staining of NeuN-positive cells in the frontal cortex  
Scale bar = 100µm
- (F) Quantification of NeuN-positive cells in the whole cortex area (top) and specifically in the frontal cortex and motor cortex (bottom). wt n=4; scr n=4, c9 n=6.
- Error bars represent SEM; n.s. non-significant; \*p<0.05, \*\*p<0.01.

**Figure 3: C9ORF72 deficient mice display normal synaptic transmission and long term plasticity in the hippocampus**

- (A) Schematic representation of the hippocampic network studied (left) and representative image of a hippocampus slice positioned on the Multiple Electrode array (MEA, right). The CA1 pyramidal neurons were excited by the stimulation of the Shaffer collaterals (red) and the synaptic responses were recorded in the CA1 stratum pyramidale and the stratum radiatum (blue).
- (B) Study of the input-output curve. The CA1 post-synaptic field potential (fPSP) amplitude and slope in response to increasing stimulation of the Shaffer collaterals.
- (C) Study of the Paired-Pulse Ratio. The CA3-CA1 synapses tend to facilitate when 2 stimulations are delivered with an inter-pulse interval of 40-200ms. No significant difference was detected between the miR-C9orf72 (red) and the control animals (white and gray). On the left, representative fPSP traces in response to 2 stimulations delivered at 20Hz in wild-type (black), C9orf72 (red) and scramble (gray) animals.
- (D) Study of long term potentiation in the CA1 area. High frequency stimulation (HFS; 2x100Hz, 1s) of CA3 Schaffer collaterals induced a long-term potentiation (LTP) of the CA1 fPSPs in all animals tested. The LTP amplitude was similar between the C9orf72 and control animals. At the top right, representative sample traces from an MEA electrode before (gray) and after the LTP induction in the C9orf72 (red) and the WT (black).

**Figure 4: Absence of motor neuron loss and motor deficits in C9ORF72 deficient mice**

- (A) Rotarod testing of C9ORF72 deficient mice at 2-5-9-12-15 and 22 months (wt n=10; scr n=12, c9 n=34). No differences of sensorimotor coordination, or motor learning were observed across trials in young or aged animals.
- (B) Open field test at 2-5-12 and 18 months of age showing total activity of C9ORF72 deficient mice and controls (wt n=10; scr n=12, c9 n=34)
- (C) Representative tracings of evoked Compound Muscle Action Potentials (CMAPs) in the gastrocnemius muscle after stimulation of the sciatic nerve. Distal Motor Latency (DML), expressed in ms, was determined by the time between the stimulus and the time to onset of a negative peak in the CMAP as shown by arrows. Both graphs present DML in ms as means  $\pm$  SEM and CMAP in millivolts, as means  $\pm$  SEM for WT, scr and C9 groups (n=8). No significant difference was observed

(D) Anterior ventral horn sections (top) of C9ORF72 deficient mice and controls at 22 months of age. Quantification (bottom) of motor neurons in L3, L4 and L5 sections (wt n=4; scr n=4, c9 n=6). Scale bar = 200 $\mu$ m

**Figure 5: C9ORF72 deficient mice show evidence of mild neuromuscular junction deficits**

(A) Hind limb grip strength measured at 2-5-9-12-15 and 22 months (wt n=10; scr n=12, c9 n=34).

(B) Representative tracings of abnormal activities used to score the EMG abnormal activities: normal activity (0)/presence of abnormal activities (1) fibrillation (2a) /positive sharp wave (2b) and intermittent activities (3). The graph present EMG abnormality score strating 0 to 4. The abnormal score was significantly increased in C9 mice compared scr (p= 0,0470) (n=8)

(C) Analysis of synaptic contact in diaphragm muscle of wild-type, miR-Scramble and miR-C9ORF72 mice. Whole-mount preparations stained with  $\alpha$ -bungarotoxin for acetylcholine receptors (nAChR) in red and with anti-neurofilament (NF) and anti-SV2 antibody for axons and synaptic vesicles in green. NMJ overlaps quantification shows no differences between synaptic contacts in miR-C9ORF72 when compared to controls. Scale bar=10 $\mu$ m.

(D) Morphological study of NMJ in *Soleus* muscle of wild-type, miR-Scramble and miR-C9ORF72 mice. MiR-C9ORF72 mice present abnormal focal axonal swellings (arrows). The histograms represent quantification of both abnormalities expressed as a percentage of the 224, 260 and 243 NMJs analyzed in miR-C9ORF72, wild-type and miR-scramble mice, respectively. Scale bar=10 $\mu$ m.

Error bars represent SEM; n.s. non-significant; \*p<0.05, \*\*p<0.01.

**Figure 6: Accumulation of p62 in the absence of reactive gliosis or TDP-43 mislocalization**

(A) Quantification Iba1 positive cells in the cortex of C9orf72 deficient

(B) Quantification of GFAP positive cells in C9orf72 deficient animals

(C) Immunostaining for TDP43 showing cytoplasmic localization in aged mice (left, arrows). The percentage of cells presenting cytoplasmic staining was not significantly different between genotypes (right).

(D) Immunostaining for P62 and Lamp1 in the frontal cortex (left) showing colocalisation of both staining (arrows). The percentage of cells presenting P62 accumulations was higher in C9ORF72 deficient mice (right).

For all experiments, wt & scr n=4; c9 n=6. Error bars represent SEM; n.s. non-significant; \* $p < 0.05$ , \*\* $p < 0.01$ .

## Supplemental Information

### Supplementary Experimental Procedures

#### Generation of C9ORF72 deficient mice and breeding

Following lentitransgenesis, GFP-positive male mice were backcrossed with females to obtain the tested F1 generation.

Mice were kept on a 12h light/dark cycle with food and water available *ad libitum*.

Transgene insertion was verified by PCR genomic DNA prepared from tail (Forward primer 5'-GACCACATGAAGCAGCACGACTTCT-3' and Reverse primer 5'-TTCTGCTGGTAGTGGTCGGCGAGCT-3').

#### C9ORF72 knock-down validation

mRNA C9ORF72 reduction was quantified by qPCR in cortex, spinal cord and muscle in animals using primers described in Table S1. Two controls genes were used to verify C9ORF72 mRNA reduction, GAPDH and OraV1. RNA was extracted using RNeasy Lipid-tissue Mini kit (Qiagen) from fresh frozen tissues and quality was verified with Bioanalyzer 2100 (Agilent).

#### Behavior tests

Prior to any behavior test, mice were allowed to acclimatize to the testing room for 20 minutes. Material was cleaned between each animal with Aniospray. Males were tested before females and material was washed with water between testing groups.

##### Forced Swim test

To look for a depression-like behavior, we tested the mice with the forced swim test. Mice were placed in a glass cylinder (height 25cm, diameter 14cm) filled with water (22°C). Immobility was recorded in a single 7 min trial period. Immobility being the time the animal stayed without any active movement.

##### Social interaction test

Social interaction was tested with the classical three-chamber test. Mice were placed in a three chamber rectangular apparatus (10cmx6cmx15cm per chamber) for a 3 stage testing protocol: Firstly, the animal freely explores the 3 chambers during a 10 min period. Then, a stranger mouse is placed in one of the lateral chambers. The time the tested mouse spends sniffing the unknown animal (stranger 1) is recorded during a 10-min period. Finally, a new mouse is placed in the opposite lateral chamber. The time the tested mouse spends sniffing this new animal (stranger 2) and the previous animal (stranger 1) is recorded for a 10-min period.



### **Habituation-Dishabituation odor assessment**

To assess whether miR-C9ORF72 mice can smell and distinguish same and different odors, mice were examined with the habituation/dishabituation paradigm as previously described (Yang and Crawley, 2009). Habituation is defined by a progressive decrease in olfactory investigation (sniffing) towards a repeated presentation of the same odor stimulus. Dishabituation is defined by a reinstatement of sniffing when a novel odor is presented. Briefly, mice were confronted too two separate non-social odors and two separate social odors for a duration of 2 minutes. Time spent sniffing each odor was manually recorded. A pre-test acclimation phase presenting a neutral odor (water) was used as baseline. Each inter-trial had a 1 min duration.

### **Morris Water Maze test**

To assess conserved spatial memory, we used the Morris water maze test. A 150cm diameter pool was filled with water (22°C) made opaque by the addition of a non-toxic, odor-free paint. The escape platform was transparent plastic and was hidden 1cm under water surface in the target quadrant. The pool was placed in a room with several visual cues. Mice were placed next to and facing the pool wall successively in north, east, south and west positions.

Latencies before reaching the platform were recorded in 4-trial sessions every day for 7 days using Anymaze tracking system (Stoeling ©). Whenever the mice failed to reach the escape platform within the 1-min cutoff period, they were placed on it for 8 sec.

The probe trial was conducted on the 8<sup>th</sup> day by removing the platform from the pool, placing the mouse next to and facing the north side. Time spent in the target quadrant was recorded in a 1-min trial.

A visible platform control was performed afterwards, displacing it in all for quadrants and measuring the time the animal took to reach it.

### **Open Field test**

To measure locomotion and anxiety-like behavior we used the open field test. The open field apparatus (50x50 cm, wall height 50cm) was placed under the Topscan (CleversysInc©, VA) videotracking system. Mice were placed in one corner and the total distance moved was recorded in a 5-min session.

### **Treadmill**

Quantification of stride number, frequency, foot pressure and gait angle information was performed with the GaitScan software (CleversysInc©, VA). Mice were forced to pace at a 10cm/sec on a treadmill. A regularity index was computed accounting for motion variations.

### **Dark and Light Chamber**

To measure social anxiety, mice were placed in a two-compartment (a dark one and a light one) chamber for duration of 5 minutes. Number of times and total time spent in the light chamber was recorded with Topscan (CleversysInc©, VA). Anxiety causes a decrease in number and duration of incursions in the light chamber.

### **Accelerated rotarod test**

Motor coordination was evaluated on the accelerated rotarod (Columbus Instruments, OH, USA). Mice were placed on a revolving beam (diameter 4cm, width 8cm, height 38cm) for 4 successive trials for 2 day. The rod was accelerated gradually from 4 rpm to 28 rpm over 2 min. Latencies before falling were recorded.

### **Hanging wire**

Mice are placed on a hanging grid (30cm height) suspended with all four limbs. Time before fall was recorded with a 2-min cutoff period in 3 separate trials. Resting time between trials was of 20 minutes.

### **Grip strength test**

The pick of muscular force is measured with Columbus apparatus. Mice are placed in apparatus and rear limbs force is recorded in 4 separate trials. Resting time between trials was of 20 minutes.

### **Immunohistochemistry**

Immunochemistry was performed on 5µm paraffin-embedded brain sections from 4 wild-type, 4 miR-Scramble and 6 miR-C9ORF72 mice. Briefly, hemi brains of wild type, miR-Scramble and miR-C9ORF72 mice (23 months) were post fixed in 4% PFA for 48h. Fixed hemi brains were then dehydrated in raising concentrations of ethanol. Dehydrated hemi brains were impregnated in paraffin and stored at RT until slicing. Leica apparatus was used to slice (5µm) brains in a sagittal plane. Slices were deparaffinated in decreasing ethanol baths. Epitope retrieval was performed in a citric acid bath warmed for 10min at 600 watts in a microwave. Sections were blocked in 5% normal goat serum (NGS), 4 % BSA, PBS-Triton 0,2% for 1h at room temperature. Sections were incubated for 48h at 4°C with following primary antibodies in PBS containing NGS/4%BSA/0.2%Triton: rabbit polyclonal anti-Iba1 (1:500, Wako Chemicals, USA), mouse monoclonal anti GFAP (1:1000, Dako Agilent, USA), mouse monoclonal anti-NeuN (1:100, Millipore, USA), rabbit polyclonal anti-TDP 43 (1:200, Proteintech, USA), mouse monoclonal anti-p62 (1:100, Abcam, UK). Secondary antibodies coupled to a fluorochrome were used to visualize immunostaining (goat monoclonal anti-mouse Alexa 555 1:1000, and goat monoclonal anti-rabbit Alexa 488 1:1000; Thermofisher Scientific, USA). Sections were incubated with secondary antibodies for 2 hours at room temperature (RT) and Dapi was used to stain nuclei. Sections were mounted using Aquapolymount (PolyScience, USA). Digital images were captured with slide scanner Axioscan (Zeiss, Germany) or with Apotome (Zeiss, Germany).

### **Neuronal loss quantification**

Neuronal loss was quantified by counting the total number of neurons in frontal and motor cortex at 2.0mm from inter-neural line (from Paxinos Atlas) using Stereo Investigator software (MBF Bioscience, USA). A threshold was previously defined and all images were analyzed using the same parameters.

### **Quantification of microglia activation and GFAP-reactive cells**

Cell count based on Iba-1 staining of microglial cells and GFAP+ astrocytes were performed on images encompassing the cortex. An experimenter who was blind to the genotype counted

each positive cell. Number of cells counted was then reported to the surface area (mm<sup>2</sup>) measured using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

### **Quantification TDP 43 and p62**

Cells displaying cytoplasmic TDP 43 were quantified in 4 20x resolution different images by an experimenter who was blind to the genotype. Number of cells counted was then reported to surface area and number of positive TDP 43 cells. Positive p62-positive cells were quantified in the same images and number of cells was then reported to surface area and number of cells in the field (DAPI staining).

### **Acute hippocampal slice preparations**

Hippocampal slices were obtained from 18 months old wild-type (n=6), C9ORF72 deficient mice (n=7) and miR-scramble (n=6) mice. Mice were anesthetized with pentobarbital (140mg/kg) and then perfused through the heart with an ice-cold “cutting” artificial cerebrospinal fluid (ACSF) solution containing (in mM) 75 Sucrose, 87 NaCl, 2,5 KCl, 7 MgCl<sub>2</sub>, 1,25 NaH<sub>2</sub>PO<sub>4</sub>, 26 NaHCO<sub>3</sub>, 0,5 CaCl<sub>2</sub> and 25 glucose, saturated with 95% O<sub>2</sub>/5% CO<sub>2</sub> (pH 7.2–7.3 and 315 mOsm/kg). Transversal slices (350 μm) were cut with a Leica VT1200S microtome (Leica Biosystems, Germany) at 0–2°C and recovered for 10 minutes in the same solution at 32°C and then for 1 hour at room temperature in the “recording” ACSF solution containing (in mM) 119 NaCl, 5 KCl, 2,5 CaCl<sub>2</sub>, 1,3 MgCl<sub>2</sub>, 9,3 KH<sub>2</sub>PO<sub>4</sub>, 25 NaHCO<sub>3</sub>, and 5 glucose, saturated with 95% O<sub>2</sub>/5% CO<sub>2</sub> (pH 7.2–7.3 and 300 mOsm/kg). For MEA recordings, hippocampal slices were transferred to an MED64 probe (Alpha MED Scientific Inc., Japan) with an inter-electrode distance of 150 μm and continuously perfused (3 ml/min) with ACSF solution saturated with 95% O<sub>2</sub>/5% CO<sub>2</sub> (pH 7.2–7.3 and 300 mOsm/kg) at 32 °C.

### **MEA recordings**

Field post-synaptic potentials (fPSPs) were recorded in the stratum pyramidale and stratum radiatum layers of the CA1 hippocampal region using a multi-electrode array (MEA) system (MED64, Alpha MED Sciences). Precisely, fPSPs were evoked by stimulation of the Schaffer collaterals/commissural pathways with biphasic current pulses (200 μs). Input-output (I/O) curve were performed using a sequence of increasing stimulus amplitude from 10 to 100 μA. fPSPs were then evoked using a stimulation intensity which elicited 40–50% of the maximum response. Paired pulse facilitation (PPF) was measured using two stimulations with an inter-pulse interval varying from 20 to 200 ms. Percentage of facilitation was calculated by dividing fPSP slope elicited by the second pulse by the fPSP slope elicited by the first pulse. For LTP protocol, two stimulations with 50 ms inter-stimulus interval were applied at a frequency of 0.1Hz. LTP was induced by a high frequency stimulation (HFS) consisting of 2 trains at 100 Hz for 1 s delivered 5 s apart at 70–80% of the intensity that evoked the maximum fPSPs. Before HFS application, a stable baseline was first established for at least 15 min. To ensure input specificity and viability of slices, a second electrode was selected in the CA1-CA2 subicular region to stimulate a second input independent from the tetanized input. The magnitude of LTP was quantified as the percentage change in the fPSP initial slope (10–40%) and the peak-to-peak amplitude taken during the 45–60 min interval after HFS. Data were filtered at 1 kHz and digitized at 20 kHz and analyzed using Mobius software (Alpha Med Scientific). Additional data analyses were performed using Microsoft Excel and

SigmaPlot. The experimenter was blind to the animal's genotype throughout the experiments for both acquisition and analysis.

### **Spinal cord analysis**

Spinal cord analysis was performed on 30 $\mu$ m paraffin-embedded sections from 4 wild-type, 4 miR-Scramble and 6 miR-C9ORF72 mice. Embedding protocol is identical to previously described protocol for brain sections. Spinal cord slices were deparaffinated in decreasing ethanol baths and lumbar motor neurons were marked with Nissl staining (Cresyl Violet). Motor neurons were quantified by an experimenter blind to genotype in Leica DM250 microscope (20x). Digital images were obtained with slide scanner Axioscan (Zeiss, Germany).

### **In vivo neurophysiological investigations**

Neurophysiological investigations were performed on 20 month-old mice. Four male and four female mice were used. Mice were kept sedated with 2-3% isoflurane delivered *via* a mask during the full procedure. The body temperature was monitored with a rectal probe and maintained at 35-37°C by placing the animals on a warming pad.

Bilateral hind limbs of all animals were examined using the Neuro-Mep-Micro electromyogram (EMG) apparatus (Neurosoft, Russia). A monopolar needle (Spesmedica, MN3512P150) was inserted subcutaneously at the base of the tail to ground the system.

Spontaneous activities were searched for with a concentric needle electrode inserted in the *Gastrocnemius* (Technomed, TE/B50600-001). The signal was bandpass filtered 20 Hz to 10 KHz. We used an EMG abnormality score scale ranging from 0 (normal) to 4 (most severe) to estimate the extent of spontaneous activities (Evans et al., 2014). Briefly, our score takes into consideration the presence of spontaneous activities, fibrillations or positive sharp waves (PSWs), intermittent multiple fibrillations and PSWs, and their bilateral localization.

To investigate the motor nerve conduction, the sciatic nerve was stimulated proximally at the sciatic notch and distally at the ankle with supramaximal stimuli of 0.2 ms through a needle electrode (Spesmedica, MN3512P150). Compound Muscle Action Potential (CMAP) was recorded by an active needle electrode inserted distally to the knee joint over the proximal portion of *Gastrocnemius* and a reference needle electrode inserted over the Achilles tendon. Distal Motor Latency (DML) was calculated from the latency of the stimulus to the onset of the negative peak of the CMAP. Baseline-to-peak amplitude measurements were used to compare wild-type, miR-Scramble and miR-C9ORF72 mice. The results from the left and the right muscle evoked responses were measured and averaged.

To assess for failure of neuromuscular transmission at the NMJ, the Repetitive Nerve Stimulation test (RNS) was performed as described in Echaniz-Laguna. A significant decrement was defined by a decrease of 10% between the first and tenth CMAP negative peak amplitude response.

### **Morphological study of NMJ and muscle in mice**

Soleus muscle, extensor digitorum longus (EDL) muscle and diaphragm were taken from mice at 5, 15 and 23 months of age after asphyxia in CO<sub>2</sub>. Whole mounts of leg specimens were fixed with 4% paraformaldehyde and diaphragms with 1% paraformaldehyde. All specimens were stained for acetylcholine Receptor (AChR) with TRITC-labeled  $\alpha$ -bungarotoxin (1:1000, Molecular Probes, Leiden, The Netherlands), for neurofilaments with a 168 kDa neurofilament antibody (1:200, 2H3, Hybridoma Bank, IA) and for synaptic vesicles

with a SV2 antibody (1:200, Hybridoma Bank, IA). Morphological analysis of the endplate was performed by confocal microscopy (Olympus FV-1000, Germany) and analyzed by ImageJ software (National Institutes of Health, Bethesda, MD, USA).

For muscle structural analysis, fresh muscle specimens were snap-frozen in liquid nitrogen cooled isopentane for cryosection or fixed in 4% paraformaldehyde (PFA) diluted in PBS for whole-mount preparations. Transversal cryosections were stained with hematoxylin-eosin (H&E) and modified Gomori trichrome stains.

### Statistical Analysis

For behavioral analysis, intergroup differences were evaluated by non-parametric Kruskal-Wallis test followed by a post hoc Dunn Test. Quantification analyses were evaluated with parametric two-way ANOVA followed by Tukey's multiple comparison test after verifying normality with D'Agostino & Pearson omnibus normality test. Electrophysiological analyses were performed with paired-t-test. All tests were performed using Prism software (Graphpad SoftwareInc). Values of  $p < 0,05$  were considered statistically significant. All data are presented as means  $\pm$ SE.

### Supplementary table S1: List of primers used for qPCR

	Sense	Reverse
OraV1	TGGAGCAGGACATATTTGACGC	AGTGGGATCATCGTAAGGAAAGT
GAPDH	AGGTCGGTGTGAACGGATTG	GGGGTCGTTGATGGCAACA
C9ORF72	CGCAGGACACCATCATCTAC	GGCTTCAAATGGAAGACCTG

### Supplementary Figure 1:

- Complementary analysis of brain functions and histology (wt n=10; scr n=12, c9 n=34)
- (A) Olfactory habituation/dishabituation test at 12 months used to measure if animals can differentiate between same and different odors, social and non-social.
- (B) Three-chamber test control at 2-5-12 and 18 months measuring social interaction by the mean time spent sniffing an object versus sniffing a mouse. C9ORF72 mice are capable of discerning between an object and a mouse.
- (C) Dark and light test to identify anxiety-like behaviors at 2-5 and 12 months. The time spent in the light chamber is measured.
- (D) Morris water maze for spatial memory at 2-9 and 15 months shows no difference in learning (left) or memory (right) in C9ORF72 deficient mice.
- (E) Representative examples of adult brains of miR-C9ORF72, miR-scramble and wild-type mice at 18 months. Scale bar =5 mm.

- (F) Macro-measurements of brain width at caudal, bregma, parietal & frontal positions (wt n=4; scr n=4, c9 n=6).
- (G) Representative sections of hippocampi of miR-C9ORF72, miR-scramble and wild-type mice at 23 months. Scale bar = 500 $\mu$ m.

### Supplementary Figure 2:

Complementary analysis of motor functions and muscles histology (wt n=10; scr n=12, c9 n=34)

- (A) Quantification of normal step number and stride length by Treadmill. No alteration could be seen with C9orf72 deficient animals
- (B) Hanging wire test measuring time to fall at 2-5-9-12 and 15 months.
- (C) Muscle characterization by ATPase staining at pH 9,4 (left) and H&E staining (right) at in 23 months old mice show not difference (n=4 per group)

Error bars represent SEM; n.s. non-significant; \*p<0.05, \*\*p<0.01.

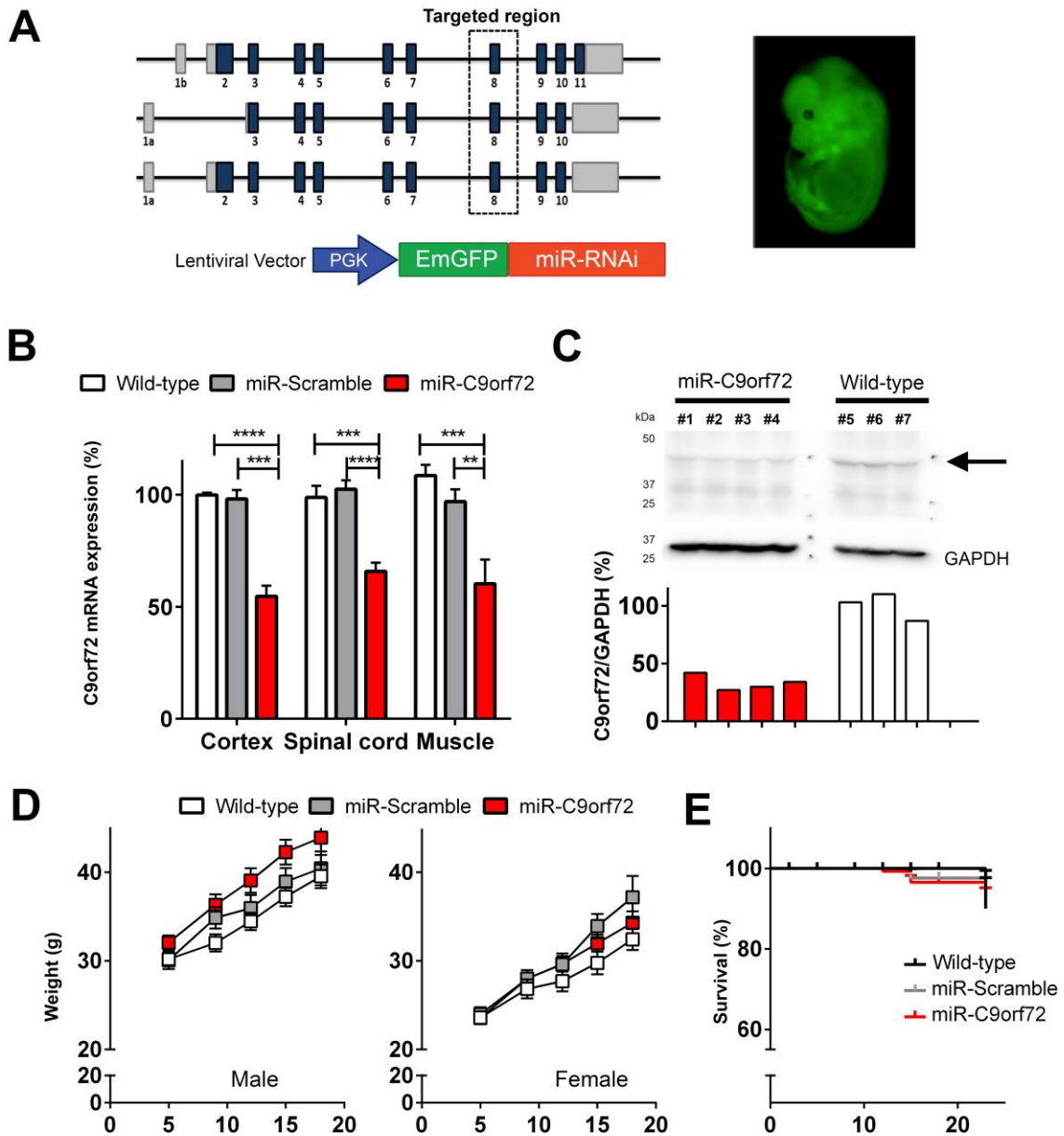


Figure 1

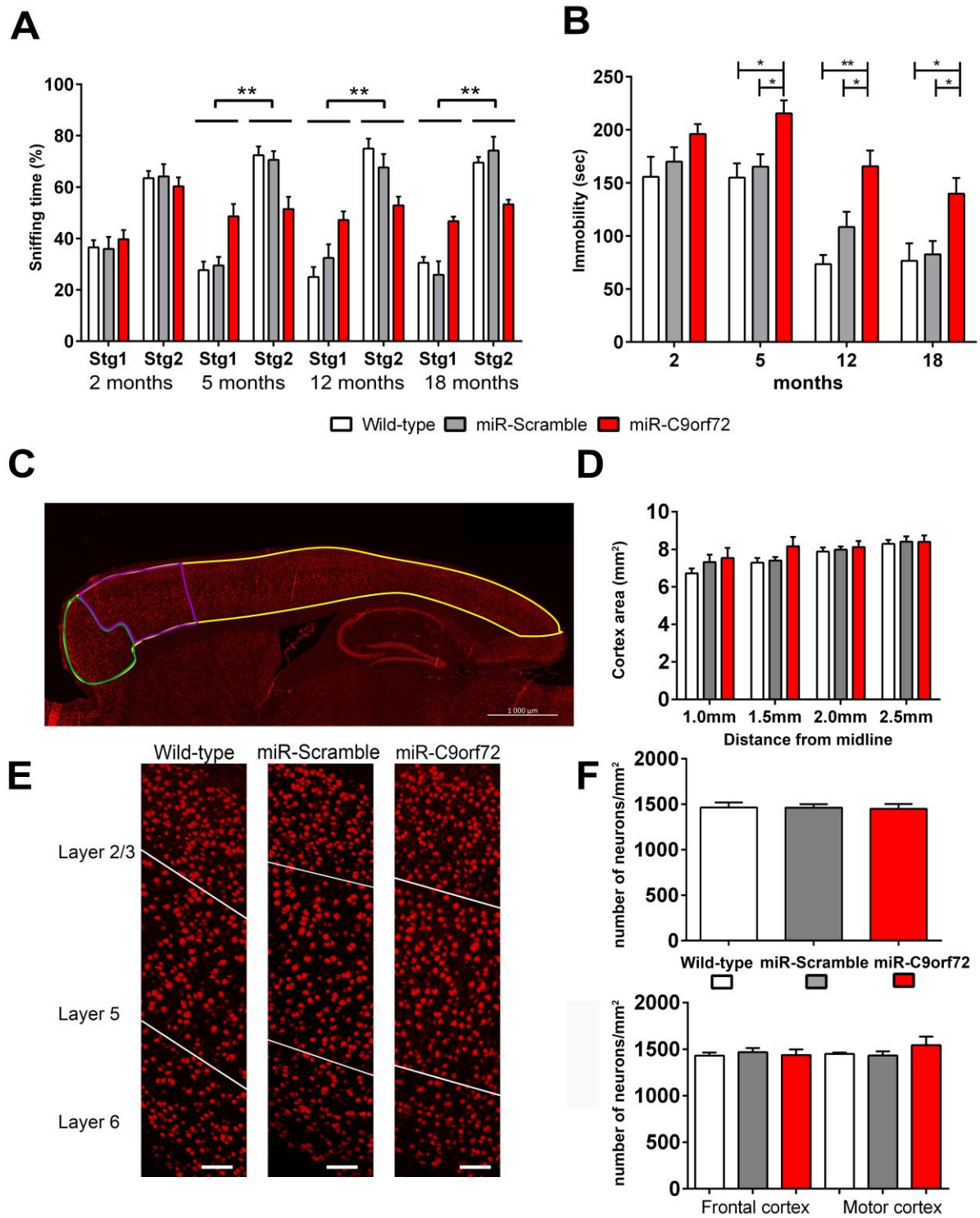


Figure 2



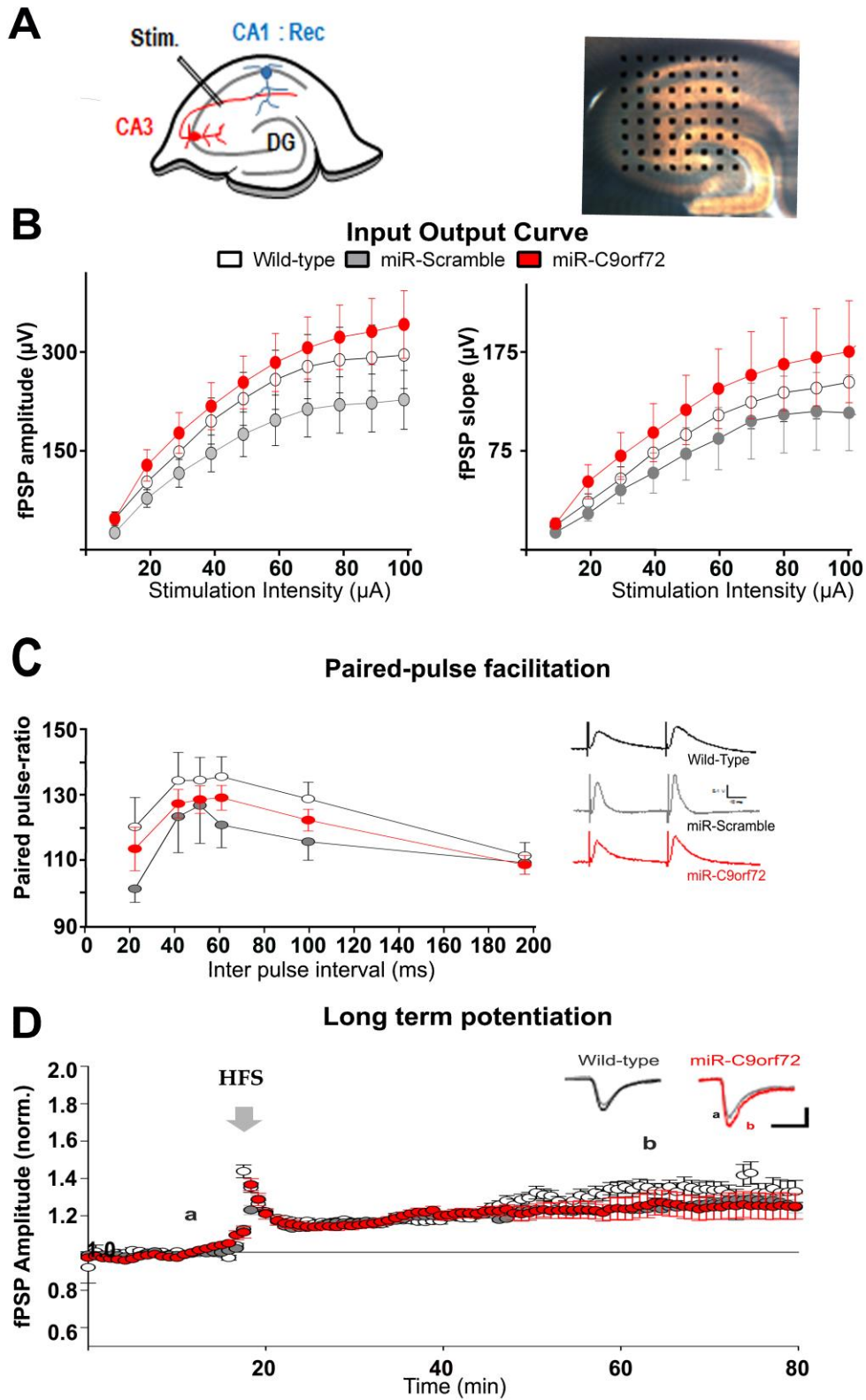


Figure 3

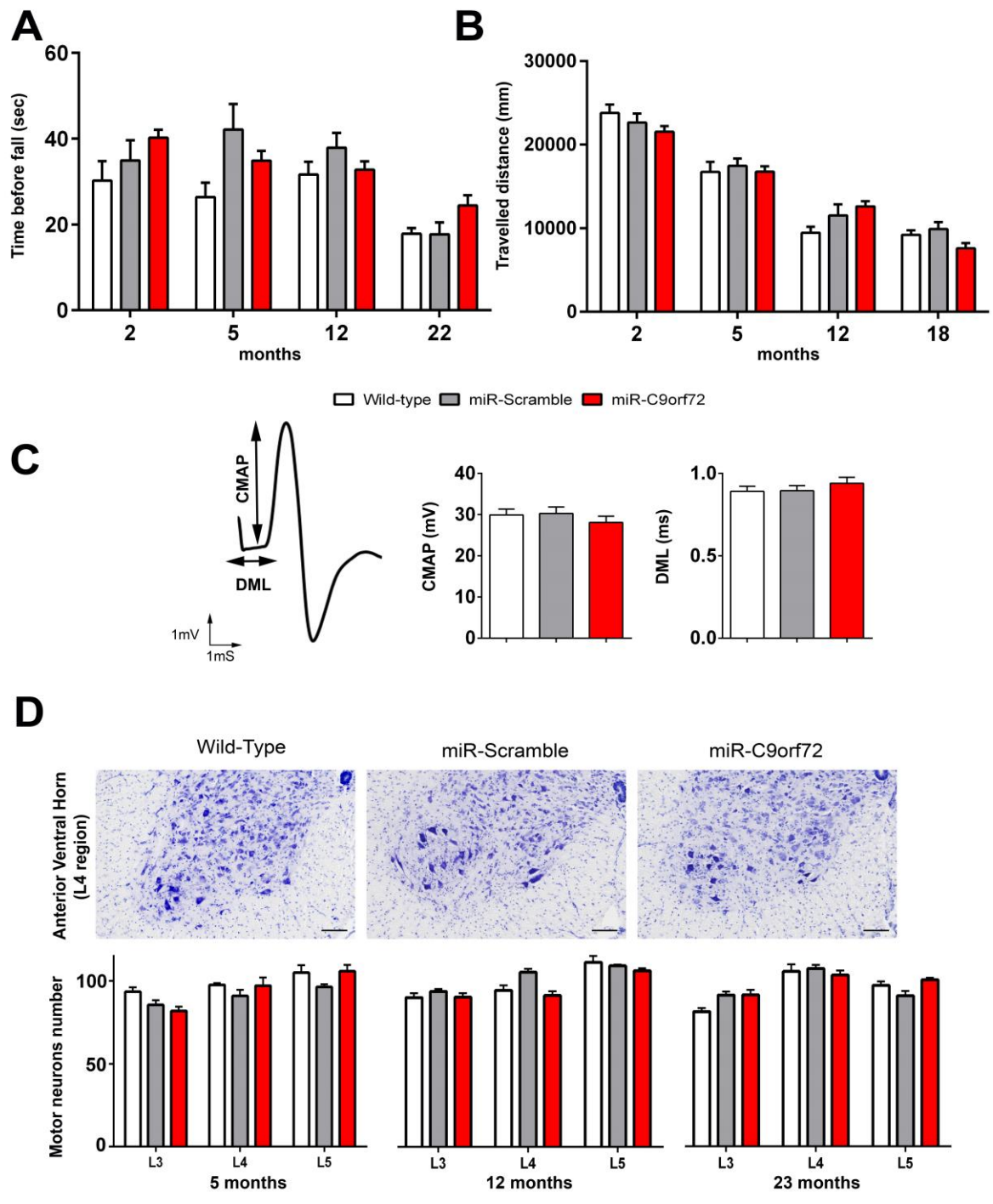


Figure 4

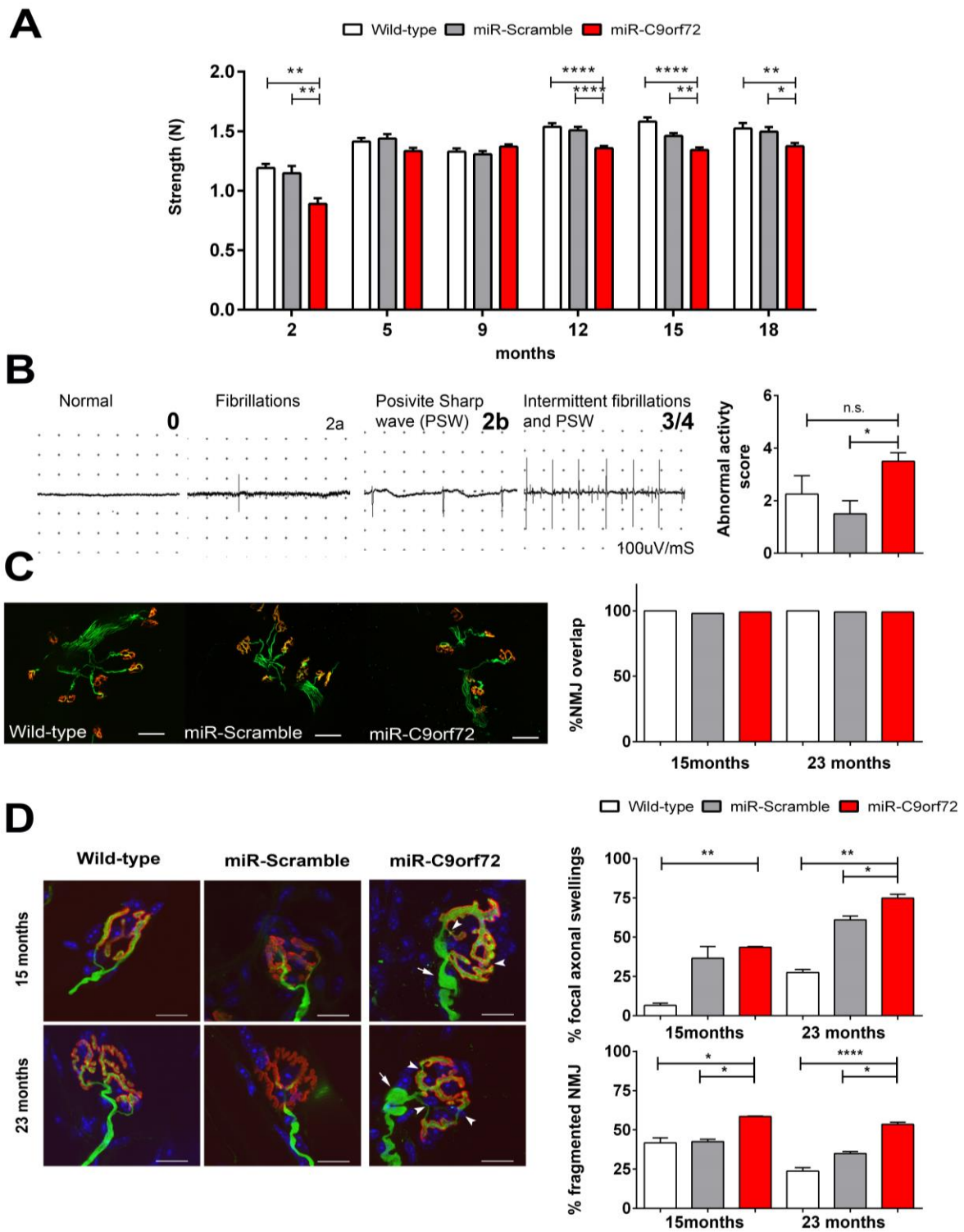


Figure 5

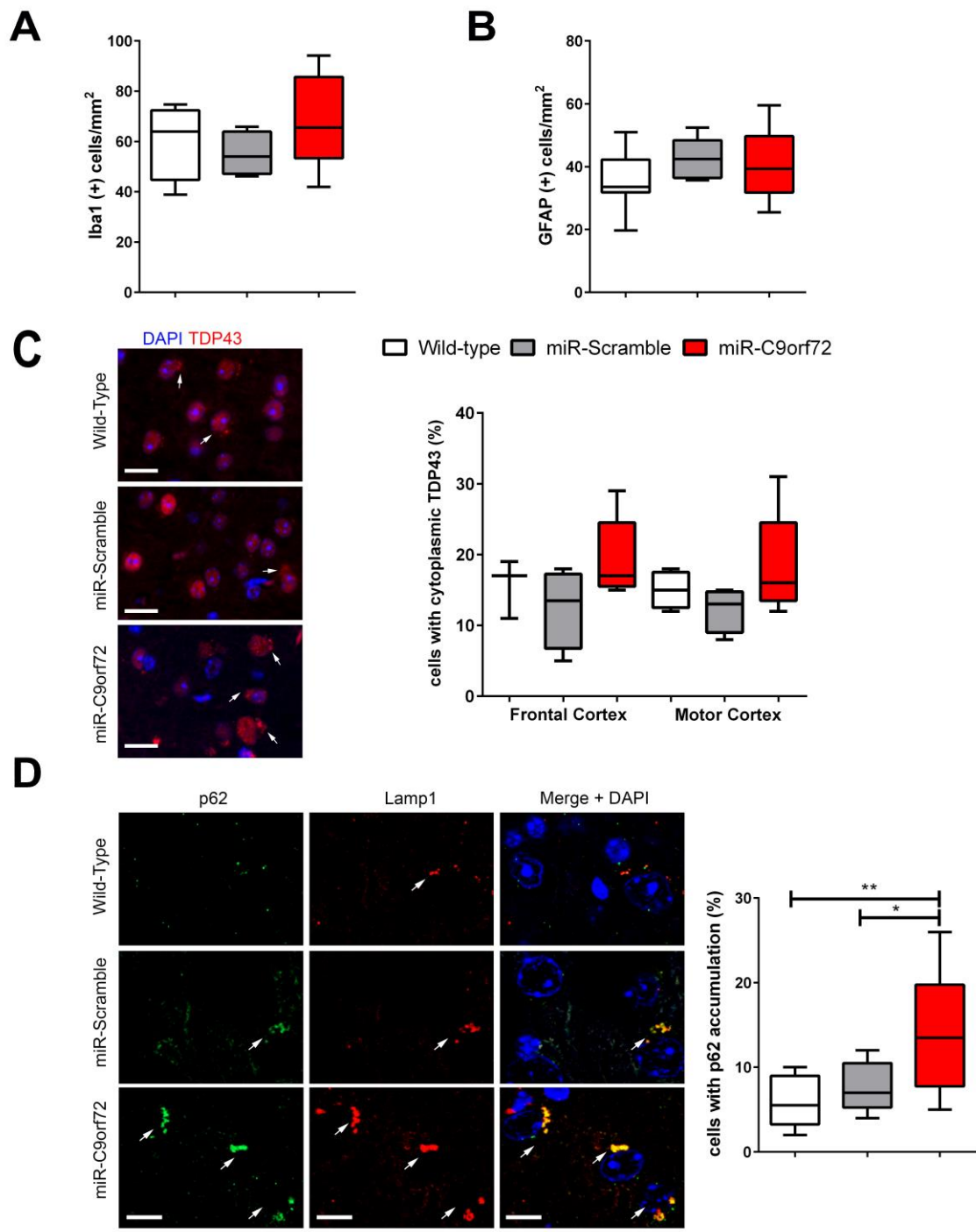
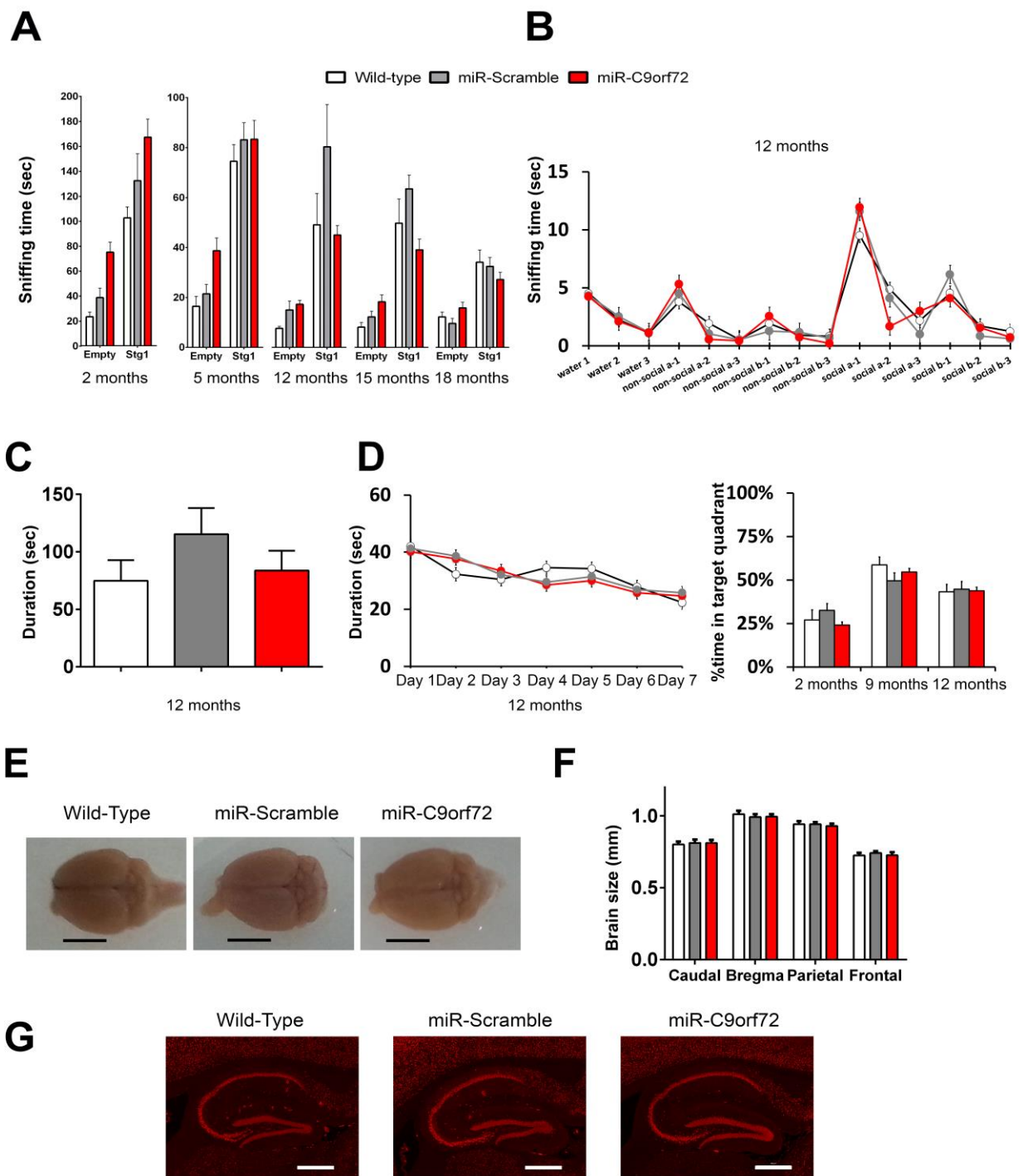
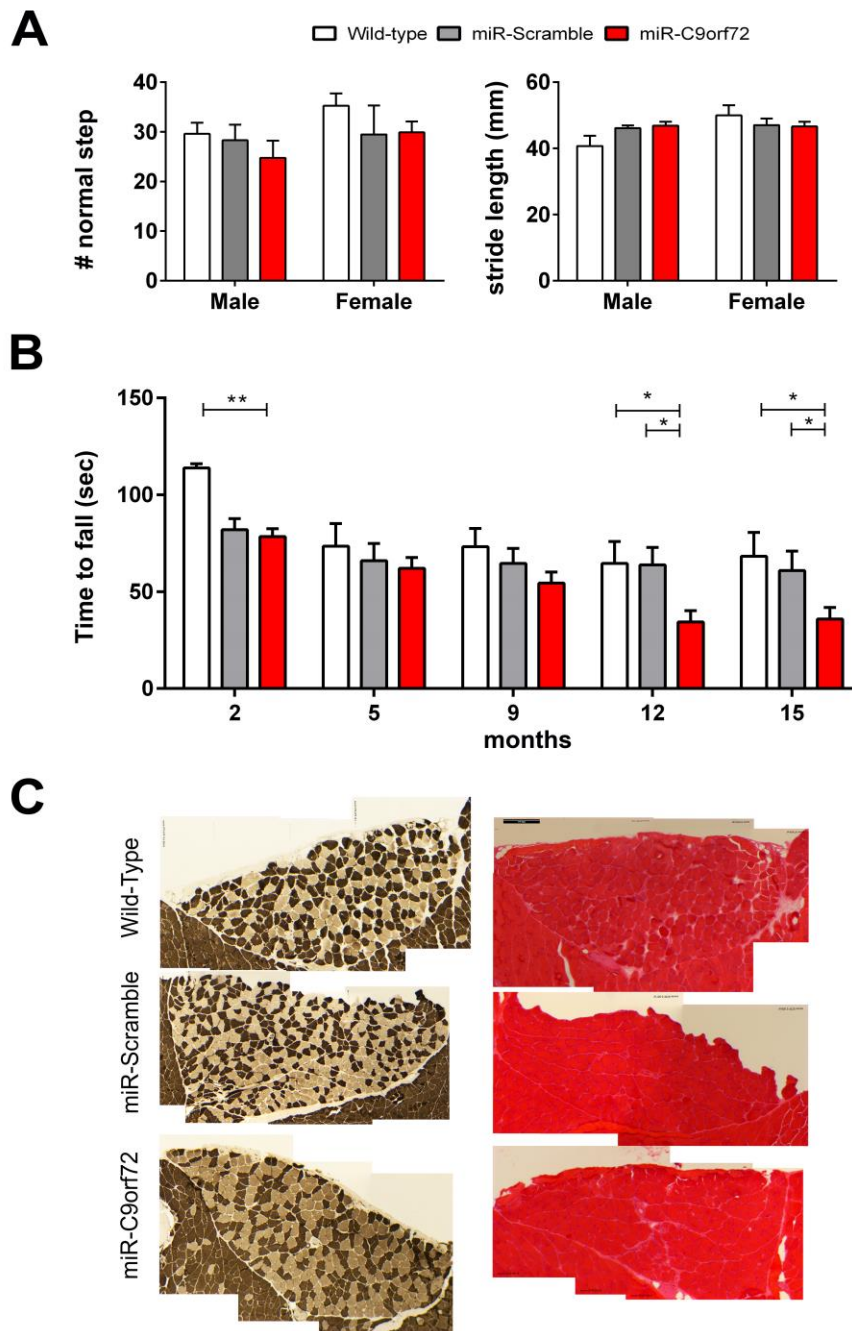


Figure 6



Supplemental Figure S1



Supplemental Figure S2

## Discussion and future perspectives

### I. Role of the C9orf72 loss-of-function mechanism in the disease course: gaining insight from the miR-C9orf72 mouse model

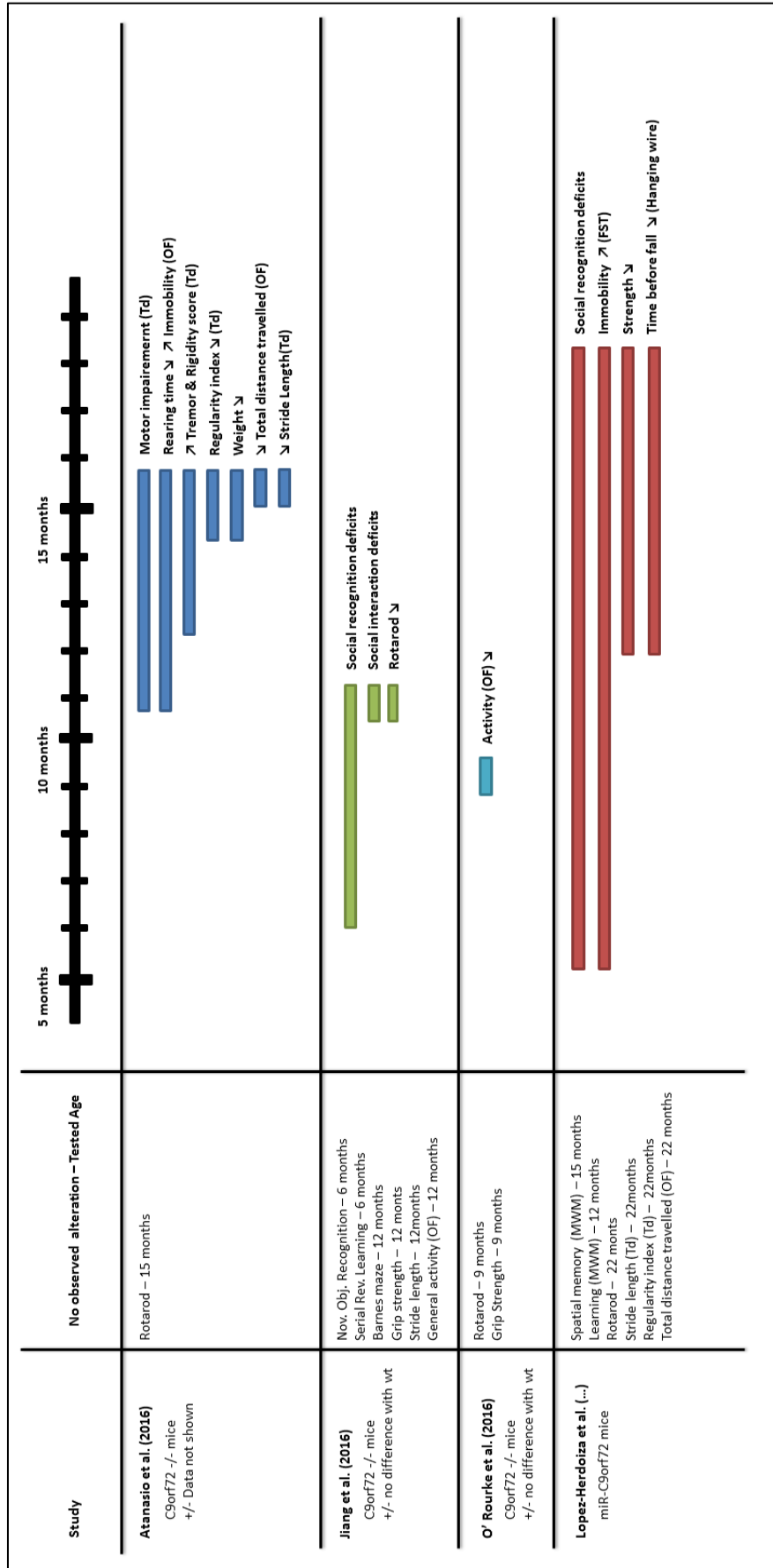
The main goal of this project was to characterize a new C9orf72 deficient mouse model in order to identify if the loss-of-function can cause abnormalities related to FTD or ALS pathology in mice. We have shown that C9orf72 deficient animals developed social interaction abnormalities without neuronal loss. Furthermore, the loss of C9ORF72 does not cause major motor deficiencies or motor neuron loss. Minor lesions in the neuromuscular junction were identified suggesting a denervation-reinnervation process. And presence of p62 aggregates in the brains of C9orf72 deficient mice suggests protein homeostasis alteration.

Since the discovery of C9orf72 as the main cause of both FTD and ALS, teams around the world have developed and characterized models intended to mimic the mutation. The presence of RNA foci was identified in patients brains (DeJesus-Hernandez et al., 2011; Renton et al., 2011) suggesting a toxic-gain-of-function mechanism, particularly since the discovery that capture of RBPs can lead to cellular death (Yang and Hu, 2016). In parallel, groups also observed a diminished expression of C9orf72 in mutation carriers, but the identification of a homozygous individual with no increased phenotype (Fratta et al., 2013) and the absence of coding mutations in the gene (Harms et al., 2013) displaced the loss-of-function hypothesis to a second plane. Shortly after the first cellular models started to appear (Almeida et al., 2013; Fratta et al., 2012), two groups showed that the hexanucleotide expansion can lead to the production of c9RAN peptides through the non-canonical RAN translation (Ash et al., 2013; Mori et al., 2013b). Rapidly, other groups showed that these c9RAN (DRPs) peptides are themselves toxic in cells and flies models, particularly those which carried an arginine unit (Mizielinska et al., 2014). These peptides were shown to be present in patients brains, but their presence did not correlate with affected regions (Mackenzie et al., 2013, 2015), bringing their effect into question.

With the appearance of mouse models, discernment of the individual contribution of each proposed mechanisms became even more complex as gain-of-function models presented contradicting results and loss-of-function models did not present a major neurodegenerative

phenotype. Overexpression of c9 HRE and c9RAN peptides through viral vectors lead to altered cognitive and motor behaviors, with neuronal loss and major histopathological hallmarks of the disease (Chew et al., 2015; Zhang et al., 2016). However, expression of the HRE at a mere physiological levels in C9orf72 BAC models lead to the presence of RNA foci, c9DPRs peptides with (Jiang et al., 2016; Liu et al., 2016) or without (O'Rourke et al., 2015; Peters et al., 2015) behavioral abnormalities. Differences in expression level between these models may explain the presence or absence of phenotype, as mice expressing endogenous levels do not develop abnormalities (O'Rourke et al., 2015; Peters et al., 2015), but overexpression of HRE will lead to a FTD/ALS-like phenotype (Liu et al., 2016). Size threshold of the repeat seems also to be an important factor in triggering disease onset and the appearance of a phenotype (O'Rourke et al., 2015). Unfortunately, a systematical analysis of behavioral alterations has not yet been established among groups, and often FTD-like anomalies are left unstudied. ALS-like anomalies are looked upon mainly using the Rotarod and the Grip Strength tests and sometimes complementary analyses measuring conductance properties, muscular alteration or neuromuscular junction anomalies are performed in the different mouse models, although these analyses are not systematically done. Studies looking into FTD or ALS anomalies in C9orf72 ablated mice showed contradicting results as well, with animals presenting no behavioral alteration (**Figure 29**) (ANNEXE 2) (O'Rourke et al., 2016) or mild alterations (Atanasio et al., 2016; Jiang et al., 2016). As presented in the introduction, most groups have chosen to consider these behavioral alterations as irrelevant due to their mild effect. Homozygous mice presented motor and social shortfalls (**Figure 29**) (ANNEXE 2) with decreased survival but not major histological hallmark of pathology (Atanasio et al., 2016; Jiang et al., 2016). Heterozygous mice presented normal survival and motor or cognitive shortfalls were not significantly different to wild-type controls. Ablation of C9orf72 specifically in neurons did not cause neuronal death or astrogliosis (Koppers et al., 2015). Interestingly, complete ablation of C9orf72 in mouse models caused major systemic autoimmune alterations (Atanasio et al., 2016; Jiang et al., 2016; O'Rourke et al., 2016; Sudria-Lopez et al., 2016). These observations raise the question of a possible role of C9orf72 protein in immunity, particularly in the endosome-lysosome maturation process. Implications of C9orf72 in immunity will be further discussed in the corresponding section.





**Figure 29** Behavior alterations in the absence of C9orf72

Summary of observed behavioral alterations in published models exploring effects of C9orf72 loss-of-function. Open Field OF; Treadmill Td

Adding to the knowledge of published mouse models, the *C9orf72* deficient mice that I characterized during my PhD present some FTD and ALS-like abnormalities without brain atrophy or motor neuron loss. The initial strategy of using miR-RNAi to knockdown *C9orf72* allowed us to create a batch of animals with a slightly variable level of gene expression, which would be comparable to variations in expression levels as seen in patient brains. Overall, miR-*C9orf72* mice had a decrease of around 40% of gene expression, which could be comparable to levels of expression in heterozygous mice. Nevertheless, when further analyzing this expression in the miR-*C9orf72* animals, we observed variability in gene expression levels such as that observed in patient cohorts (Ciura et al., 2013; DeJesus-Hernandez et al., 2011). Due to the fact that the behavioral phenotype we observed was mild, we could not establish a direct correlation between expression level and the observed alterations. FTD-like abnormalities were observed from a relatively young age (5 months), but ALS-like anomalies were harder to identify, with the only signs appearing at 9 months in male mice and 12 months in female mice and no major phenotypical evolution. Contrary to other published models, in these mice the immune response was not studied, although we did observe mice occasionally with increased spleen size suggesting a splenomegaly and altered immune response (*data not shown*). However, I did not explore this further as I focused on characterizing the nervous system.

As the aim of this thesis was to characterize and validate a new mouse model of *C9orf72* loss-of-function, the question of its validity as a suitable model for studying underlying mechanisms of FTD or ALS remains. The *C9orf72* mutation can act through different mechanisms leading to neuronal death, but the specific or independent role played by each of these mechanisms is not yet established. Different models mimicking one or several of these mechanisms have not been able to fully reproduce a phenotype corresponding to c9FTD/ALS, and thus, the way through which the expansion will trigger pathology remains elusive. Both RNA foci and c9DPR proteins overexpression lead to decrease survival and other disease hallmarks in several disease models (Chew et al., 2015; Jiang et al., 2016; Liu et al., 2016; Zhang et al., 2016), but physiological expression did not cause a major phenotype (O'Rourke et al., 2015; Peters et al., 2015). On the other hand, *C9orf72* deficiency alone did not cause decreased survival in mice, and complete ablation of the gene caused an important autoimmune response (Atanasio et al., 2016; O'Rourke et al., 2016). So far, available information from several models states that RNA foci and DPRs are toxic elements to the cell,

but the extent at which physiological expression causes phenotypical alterations requires further research, as most models overexpress these toxic products.

All things considered, the knowledge acquired from the characterization of the miR-C9orf72 mice brings an important insight into the field. In effect, C9orf72 deficient mice develop behavioral abnormalities that are not explained by neuronal or motor neuron death or altered hippocampal synaptic transmission, bringing the question of what triggers this altered behavior. Additionally, the observed alterations at the NMJ level suggest the miR-C9ORF72 mice develop initial stages of an ALS-like alteration, as seen in early stages of ALS pathology (Dupuis and Loeffler, 2009). Finally, mice presented an increased p62 staining that colocalizes with staining of the lysosomal pathway marker Lamp1, suggesting a possible alteration in protein degradation pathways, particularly the autophagy pathway or the endosomal-lysosomal pathway (Bitto et al., 2014; Pankiv et al., 2007). Together, these data suggest a not so secondary role of the C9orf72 loss-of-function mechanism, but rather an effect in which the decrease in the functional protein levels will render the cell more susceptible to HRE-related stress. From this hypothesis it is possible to assume that the convergence of added toxicities, both the RNA-toxic expansions and the DPRs and probably also aging, in an already feeble cell, will lead to the final neurodegenerative process. Backing up this hypothesis, is the idea that loss of autophagy leads to a neurodegenerative phenotype (Hara et al., 2006; Komatsu et al., 2006; Wong and Cuervo, 2010) and alterations in this pathway, as those possibly caused by the C9orf72 loss-of-function (Webster et al., 2016), could be keys triggers in neurodegeneration. Furthering reinforcements are the observations of altered endosomal-lysosomal maturation in the microglia of C9orf72 knockout mice (O'Rourke et al., 2016). Also the fact that mutations causing familial forms of both FTD and ALS are related to protein degradation and autophagy pathways (Le Ber et al., 2013; Skibinski et al., 2005). This is the case for mutations in *CHMP2B* gene which codes a protein belonging to the ESCRT but also in the *VCP* gene coding for protein implicated in autophagy, proteasome and endosomal degradation pathways, mutations in *UBQL2* gene that codes for a protein in charge of targeting proteins to the proteasome degradation pathway and mutations in *SQSTM1* coding for p62 protein, which was largely discussed in Introduction - Chapter I.

Although a multiplicity of observations point toward the autophagy pathway, preclinical trials of both chemical and genetic interference with autophagy pathways did not yield major benefits in lifespan or muscle denervation in ALS mouse models (Dewil et al., 2007; Gould et al., 2006; Rouaux et al., 2007) questioning the implication of this pathway. Interestingly, in

most of these trials, complete motor neuron rescue was observed suggesting that motor neuron itself is not the primary cause of death, but that autophagy has a direct influence in motor neuron survival (Dupuis and Loeffler, 2009). From this point, several cell types including skeletal muscle and glial cells also contribute and modulate disease evolution (Boillée et al., 2006a; Dupuis et al., 2009), through identical or different additional mechanisms other than autophagy. Thus, a convergence of factors will lead to neurodegeneration itself and although the C9orf72 deficient mice do not present the RNA foci and c9DPRs proteins, they remain a useful tool for uncovering alterations in cellular homeostasis due specifically to the loss of C9orf72 function.

## II. Behavior evaluation of mice models and correlations to clinical pathology

Studying behavioral alterations in mice can be quite challenging, particularly when looking into executive functions and higher cognitive processes. Contrary to motor deficiency, where a disability can be measured, cognitive abnormalities in mice are subjected to multiple interpretations.

Studying FTD related behaviors in mice can be complicated, particularly when considering that mice do not have a frontal cortex as complex as primates. Thus, finding the right test to identify an effect evoking a human symptom remains a challenge (**Table 5** and **Figure 30**). Nevertheless, being able to identify cortical alterations in mice remains a crucial step. Functional behavior alterations serve as a quantitative marker for human disease symptoms and can be used as a preclinical tool for evaluating new treatments.

**Table 5** Behavior assessment of mouse models

Clinical observation	Related brain structure in patient	Homologous structure in mice	Behavioral test
<b>Disinhibition</b>	Orbitofrontal cortex	Amygdala	Elevated plus maze Light/Dark chamber Go/No-Go test 5-Choices serial reaction time task
<b>Depression</b> <b>Inertia</b>	Prefrontal cortex Basal ganglia		Tail suspension Forced swim test
<b>Apathy state</b>	Frontotemporal cortex Tempoparietal networks	Anterior cingulate cortex Hippocampus Amygdala	Observational fear learning Three-chamber test
<b>Stereotyped behavior</b>	Prefrontal cortex Striatum	Striatum	Grooming and digging time
<b>Dietary changes</b>	Hypothalamus Anterior cingulate cortex	Hypothalamus Amygdale Anterior cingulate cortex	Sucrose preference Alcohol seeking
<b>Mental inflexibility</b>	Frontal-thalamic loop		Reversal learning
<b>Episodic and spatial memory</b>	Hippocampus Parietal lobe	Hippocampus  Amygdala	Morris Water Maze Barnes Maze Novel object recognition Fear learning

Importantly, the anatomical structures and networks implicated in neurodegeneration are highly conserved between rodent and humans (Lu et al., 2012), in particular, the salience network, which is highly affected in FTD (Boccardi et al., 2005; Schroeter et al., 2008; Seeley et al., 2009, 2008). This network is anchored in the insula, and includes the anterior cingulate cortex, ventral striatum, amygdala, dorsomedial thalamus, hypothalamus, and brainstem nuclei including the ventral tegmental area, periaqueductal gray and substantia nigra (**Figure 31**) (Seeley et al., 2007). It controls primitive functions such as pain, hunger, reward, motivation and fear responses (Roberson, 2012). Despite the lack of an evolved prefrontal cortex, the mouse brain presents the anatomical structures that are affected in FTD, allowing the study of the pathology in such a model.

From this point of view, studying the diverse symptomatology of FTD in mice may seem particularly complex. As research in other degenerative diseases has shown, clinical aspects can be comparable to specific mouse behaviors (Crawley, 2007). In FTD, by focusing on clinical presentation only, three behavior-types with common neuroanatomical regions can be studied: social dysfunction, repetitive behavior and emotional behavior (Roberson, 2012).

Social dysfunction is one of the most prominent features of FTD, with patients presenting decreased social interest and lack of empathy. In mice, social interaction and

Author / ref	target gene	nature of tg	protein aggregates	social behavior	disinhibition	depression	memory	other behavior	motor impairments
Yin 2010	PGRN	KO	ND	3 chamber test	elevated + maze	tail suspension forced swimming	MWM	ND	No
Kayasuga 2007 / Ghoshal 2012	PGRN	KO	ND	resident-intruder	open field		MWM	ND	No
Filiano 2012	PGRN	KO + haploinsufficiency	Yes	3 chamber test, tube test	ND	ND	MWM	cued fear conditioning	No
Petkau 2012	PGRN	KO	No	resident-intruder	open field	forced swimming	MWM, NOR, passive avoidance	ND	Yes
Tsai 2010	TDP-43	WT overexpression in cortex + hippocampus	Yes	ND	ND	ND	MWM, NOR, fear conditioning	ND	Yes
Swarup 2011	TDP-43	A315T G348C	Yes	ND	ND	ND	barnes maze, passive avoidance test	ND	Yes
Tatebayashi 2002 / Egashira 2014	tau	R406W	Yes	ND	LDB	forced swimming	contextual fear conditioning	cued fear conditioning	No
Dawson 2007	tau	N279K	Yes	ND	ND	ND	Radial arms water maze	ND	Yes
Lambourne 2007 / Reichelt 2013	tau	V337M	No	ND	ND	ND	ND	5-CSRTT, executive function	No
Levenga 2013	tau	P301L	Yes	ND	ND	ND	contextual fear conditioning	ND	Yes
Cook 2014	tau	neuronal, forebrain specific P301L	Yes	ND	open field, elevated + maze, LDB		contextual fear conditioning	cued fear conditioning	ND
Rodriguez-Ortiz 2013	VCP	Neuronal A232E	Yes	ND	ND	ND	MWM, NOR, fear conditioning	ND	ND

**Figure 30** Mouse models of FTD and associated behavioral assessments

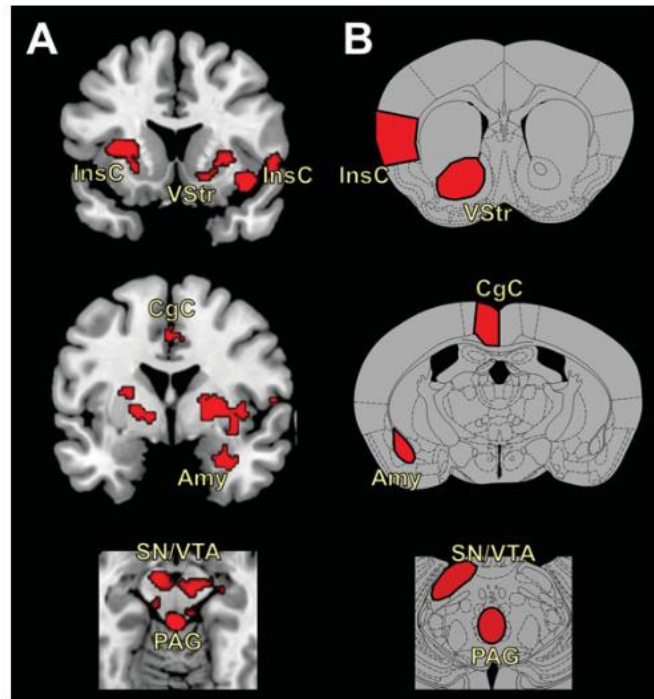
red=impaired behavior, green=normal behavior of the transgenic mice. MWM=Morris Water Maze, NOR=Novel Object Recognition, 5-CSRTT=5-Choices Serial Reaction Time Test, LDB=Light/Dark Box, ND=Not Determined.

empathy are commonly measured by the three-chamber test. FTD mice will present a lack of interest when exposed to an unknown animal (Chew et al., 2015; Ghoshal et al., 2012; Jiang et al., 2016; Kayasuga et al., 2007; Yin et al., 2010). The observational fear conditioning or the empathic chamber tests also can identify lack of empathy in mice. In both tests, the observer mouse can see a demonstrator mouse receiving mild electrical foot shocks. FTD model mice will not react to the demonstrator mouse reaction even when they are capable of recognizing the painful stimulus (Jeon et al., 2010). Patient (Rankin et al., 2003) and FTD mice models (Lindzey et al., 1961) present social dominance loss, and it can be studied in the tube test assay.

Repetitive behavior is a core feature of FTD diagnosis criteria (Crawley, 2007; Jeon et al., 2010; Langford-Smith et al., 2011; Lindzey et al., 1961; Miller et al., 1995; Rankin et al., 2006, 2003). In mice, repetitive behavior corresponds mainly to extensive grooming, increased digging and somersaults or climbing on the cage walls (Shmelkov et al., 2010). Furthermore, dietary changes are commonly seen in patients. Altered food consumption has not been explored in FTD mouse models. Repetitive behaviors and dietary changes can be studied using a HomeCage scan recording through several days.

Finally, emotional behavior impairment in patients is seen in negative emotions such as fear, anger and disgust (Werner et al., 2007). Fear conditioning paradigms allow studying fear response in mice, and altered responses correlate with amygdala atrophy (Hofer et al., 2008). And depression-like behaviors such as the forced swim test or the tail suspension test allow to identify an emotional impairment.

Memory alterations have been commonly studied in FTD mouse models giving contradicting results (Filiano et al., 2013; Ghoshal et al., 2012; Petkau et al., 2012; Swarup et



**Figure 31** Saliency network in human and mice

(A) Regions of reduced connectivity in FTD patients and (B) schematic representation of homologous brain regions in coronal sections of mice brain. *Adapted from Roberson et al. (2012)*

al., 2011; Tsai et al., 2010; Yin et al., 2010). In FTD patients hippocampal function and episodic memory are mostly spared, making memory preservation a key factor in differentiating FTD from AD (Rascovsky et al., 2011).

Characterization of animal behavior is limited by the complexity of implementing most of existing assays. As salience network abnormalities encompass different structures such as the amygdala or the hippocampus, several complementary tests should be performed to identify alterations. Experimenters should also consider the important number of animals (at least 15 per group) to account for group variability. Animals are capable of learning different paradigms, and a habituation-bias can appear when the mice are tested multiple times throughout aging, possibly diluting phenotypical alterations. The physical capacity of the mouse to perform the test should also be carefully estimated, as behavioral tests require sensorial and motor capacities. For instance, olfactory integrity is essential for social interaction assessment. Furthermore, compensatory molecular mechanisms and relatively short lifespan of laboratory mice can prevent the development of pathological phenotypes or cover up phenotypes that otherwise would become pathological.

Regardless of the assessment used to identify a particular FTD-related alteration, careful notice should be taken regarding conflicting factors when interpreting behavioral results. As mice models can develop motor dysfunction (Hochgräfe et al., 2013; Schindowski et al., 2006; Swarup et al., 2011; Tsai et al., 2010), it would be inadequate to claim an apathy-like behavior when the animal no longer capable of exploration (Jucker, 2010).



### **III. Hypothesis of underlying mechanisms driving neurodegeneration in c9FTD/ALS**

Although my PhD work did not specifically explore underlying cellular and molecular pathways leading to neurodegeneration in c9FTD/ALS, I would like to explore some hypothesis from the literature and from my own observations and address some questions.

#### **1. Protein homeostasis, aggregation and TDP43 pathology**

As most neurodegenerative diseases present some type of protein aggregates, mechanisms leading to this aggregation are commonly studied. Although many advances have been made in the past years, questions remain yet to be answered, as we still do not know whether these aggregates are rather neuro-toxic or rather neuro-protective (Chapter I). As protein degradation is a key step of protein homeostasis, disturbances at this level could lead to the accumulation of proteins. The brain may be particularly sensitive to these type of alterations since there is a continuous protein turnover of ~7 grams in a daily basis for this organ, and with aging, eliminatory pathways are decreased (Kress et al., 2014). If these aggregates are toxic, when does the toxicity starts and until what point is the cell capable of reparation? Does the toxic effect derive from sequestration of other proteins or from other mechanisms? What is the role played by aging in protein homeostasis?

Formulating a single answer for any of these questions would be unreasonable, as observations vary according to the studied protein aggregates. However, as mechanisms leading to protein aggregation may be analogous or correlated in different neurodegenerative diseases, understanding how one protein aggregates may give insight into other aggregating forms. This is the case in ALS cases with SOD1 mutations where toxicity derives rather from aggregating forms than by the loss of physiological protein function (Eftekharzadeh et al., 2016) showing that aggregation in this case is rather neuro-toxic. In effect, SOD1 knockout models do not develop motor deficits, whereas overexpression of the mutated form of the protein will cause mice to develop ALS-like symptoms (Philips and Rothstein, 2015). On the other hand, TDP43 toxicity due to aggregation is not as clear. TDP43 mislocalization and aggregation is a common feature of sporadic and familial cases of ALS and FTD patients (Burrell et al., 2016; Mackenzie et al., 2009; Neumann et al., 2006). Aggregated TDP43 commonly colocalizes with ubiquitin markers suggesting alterations in the proteasome

degradation pathway and mutations leading to FTD or ALS often belong to genes involved in protein degradation (*SQSTM1*, *CHMP2B*...) (Chapter II). Furthermore, mutations in TDP43 will lead to the presence of mutant peptides forms with longer protein half-life than wild-type forms (Chen-Plotkin et al., 2010; Ling et al., 2010) leading to abnormal protein stability prone to seeding and accumulation, and overexpression of wild-type TDP43 also aggregates (Xu et al., 2010; Ash et al., 2010). Examination of ALS sporadic patients with unusual long disease duration (10-20 years) showed presence of a relative low number of TDP43 inclusions in spinal cords (Nishihira et al., 2008) suggesting a rather selective neuronal degeneration of cells bearing TDP43 inclusion but could also suggest an initial lower amount of TDP43 aggregates, thus explaining the prolonged survival. Conversely, targeted reduction of TDP43 aggregates did not reduce or prevent cellular death in *in vitro* models (HeLa cells) (Liu et al., 2013). Moreover, knockdown of progranulin, the second most common FTD-causing gene in mouse cortical neurons induced TDP43 mislocalization to the cytoplasm resulting in enhanced vulnerability to stressors (H<sub>2</sub>O<sub>2</sub> and NMDA) (Guo et al., 2010). The loss of PGRN lead to the abnormal cleavage, ubiquitylation and accumulation of TDP43 without aggregation (Salazar et al., 2015). Thus, it seems that TDP43 aggregations may not be the main causative factor of cytotoxicity but rather its depletion from the nucleus (Liu et al., 2013).

## 2. Microglia C9orf72 implications

The function of the C9orf72 protein has not been fully elucidated, but observations in mice models with complete gene ablation argue for a function in immunity and inflammation (Atanasio et al., 2016; Burberry et al., 2016; O'Rourke et al., 2016). C9orf72 reduction seems to affect monocyte-macrophage differentiation and thereby hamper host inflammatory responses (Rizzu et al., 2016), and at least part of this effect appears to be caused by late endosomal-lysosomal maturation defects. Effectively, a group recently showed altered endosomal-lysosomal maturation in microglia of C9orf72 knockout mice suggesting this effect will lead to the diminished ability of microglia to clear aggregated proteins and/or alter their immune responses (O'Rourke et al., 2016).

In FTD, mutations in *PGRN*, one of the main disease-causing genes, lead to an inflammatory response caused by the protein loss-of-function (Pickering-Brown, 2007). *PGRN* is expressed in cortical and hippocampal pyramidal neurons, in cerebellar Purkinje cells (Daniel et al., 2000), in motor neurons (Ryan et al., 2009) and in activated microglial

cells (Mackenzie et al., 2006). Progranulin binds to cortical neurons through Sortilin 1 receptor which in turn, endocytosis and delivers progranulin to lysosomes (Hu et al., 2010). Progranulin is required to maintain lysosomal function in cells (Belcastro et al., 2011; Hu et al., 2010; Lui et al., 2016; Zhou et al., 2015), but can also be secreted from cells to act as a neurotrophic factor, having a major role in inflammation processes (Cenik et al., 2012). In effect, progranulin regulates TNF/TNFR signaling pathway (Jian et al., 2013; Tang et al., 2011), promotes CD4+ T cell differentiation and promotes antibody diversity in B cells (Puga et al., 2012; Tang et al., 2011). Interestingly, homozygous *PGRN* mutations lead to the development of neuronal ceroid lipofuscinosis autoimmune disease, whereas the *PGRN* haploinsufficiency causes FTD pathology. *C9orf72* knockout mice also present this phenotypical curiosity. *C9orf72* knockout in mice causes an increase autoimmune response with similarities to systemic lupus erythematosus (Atanasio et al., 2016; Burberry et al., 2016; O'Rourke et al., 2016; Zhen et al., 2005) whereas data obtained from the miR-*C9orf72* mice, with only a partial decrease in *C9ORF72* expression, showed a FTD-like behaviors appearing in mild adulthood. These observations are interesting because both *PGRN* and *C9orf72* may drive pathogenicity in a dose-dependent manner, causing different conditions according to the level of expression. Then again, mice are often incomplete models of diseases (Jucker, 2010). Even more in the case of the *C9orf72* mutation, where differences in protein isoforms between human and rodents will limit observations accounting for protein function or the lack of it (*refer to nucleocytoplasmic shuttling section*).

A remaining question is the fact that in contrast to *PGRN* patients who do not develop motor symptoms, patients with *C9orf72* mutations can develop ALS pathology, but both *PGRN* and *C9orf72* patients present TDP43 inclusions (Cairns et al., 2007; DeJesus-Hernandez et al., 2011; Renton et al., 2011). So far this question remains unanswered, and studies from patient samples and from animal models have not allowed for the identification specific pathways. Nevertheless, it seems probable that common altered pathways in both *PGRN* and *C9orf72* pathology would be key for the development of FTD, and additional effects of the *C9orf72* mutation uncommon to *PGRN* pathology will rather drive motor neuron disease. Effectively, observations from the miR-*C9orf72* mice showed development of mainly FTD-like anomalies. As both progranulin and *C9orf72* loss of function seem to alter microglia through lysosomal-function related deficits, the FTD phenotype, at least in mice, could be driven by these alterations. Additional effects of c9DPRs, RNA foci or other strains of TDP43 on microglia, neurons, and all components of the neurons system would have further impact in appearance and progression of ALS.

### **3. ALS/FTD mechanisms not directly related to the C9orf72 loss-of-function... or are they?**

#### **3.1. Nucleocytoplasmic transport**

Studies have recently suggested that C9orf72 disease pathology might arise from disturbed nucleocytoplasmic shuttling, mainly lead by gain-of-function toxicities from RNA-products or c9DPRs (Boeynaems et al., 2016; Freibaum et al., 2015; Jovičić et al., 2015; Zhang et al., 2016). However, DPRs proteins are not easily detected in brains and spinal cord of FTD/ALS patients, questioning their implication in neurodegeneration of affected zones (Davidson et al., 2015, 2014; Gomez-Deza et al., 2015; Mackenzie et al., 2015). To answer this question, studies in human samples are needed to connect toxicity from the HRE to the observed alterations. But as altered nucleocytoplasmic shuttling seems to be frequent in ALS pathology, as has been suggested by SOD1 models (Zhang et al., 2006), this pathway seems fundamental to explore.

Although previous studies merely looked into gain-of-function toxicities, the effects of the C9orf72 loss-of function could also be of interest in the context of nucleocytoplasmic shuttling. In effect, levels of mRNA transcript variant 1, which encodes the short protein isoform localizing to the nuclear envelop (Xiao et al., 2015), are reduced in C9orf72 expansion carriers (Blitterswijk et al., 2015). This reduced expression has been correlated to decreased survival in patients, suggesting that alterations in the level of protein expression may impair a yet unknown nuclear function. The observation that C9orf72 short protein isoform colocalizes with importin subunit  $\beta 1$  and RanGTPase at the nuclear envelop (Xiao et al., 2015) supports this idea. Accordingly, in ALS cases, the C9orf72 short isoform, importin  $\beta 1$  and RanGTPase presented cytoplasmic rather than nuclear staining and correlated with TDP43 mislocalization to the cytoplasm. Members of the importin  $\beta$  family are karyopherins capable of protein transport into the nucleus by binding to specific nuclear localization sequences (NLS) (Mattaj and Englmeier, 1998). Interestingly, members of this family are down regulated and relocalized to cytoplasm in the frontal cortex and cerebellum with aging (Mertens et al., 2015), and this down regulation has also been observed in ALS spinal motor neurons (Xiao et al., 2015). Furthermore, nuclear import of TDP43 involves interaction with Importin $\beta 1$ , and is dependent on RanGTPase (Ito and Suzuki, 2011; Nishimura et al., 2010; Ward et al., 2014). Disruption of Importin  $\beta 1$  causes cytoplasmic accumulation of TDP43

(Nishimura et al., 2010). On the same line, RanGTPase protein level alterations may explain TDP43 pathology since impairment of RanGTPase mediated nuclear trafficking correlates with mislocalization of TDP43 to the cytoplasm, before the onset of neurodegeneration in progranulin knockout mice (Nishimura et al., 2010; Ward et al., 2014). Additionally, TDP43 can regulate RanGTPase expression, suggesting that disruption of either nuclear TDP43 localization or RanGTPase expression can influence each other enhancing any toxic effects of TDP43 nuclear depletion (Jovičić et al., 2016; Ward et al., 2014). Finally, the ageing process seems to progress through gradual regression of RanGTPase mediated nuclear trafficking competency (Nagai and Yoneda, 2013). As aging has been related to defects in the nuclear pore (D'Angelo et al., 2009; Fox and Tibbetts, 2015), alterations in nucleocytoplasmic shuttling by either c9RAN peptides, RNA foci or the lack of C9orf72 short isoform may alter the cell homeostasis and enhance the aging process, leading to a degenerative phenotype. Unfortunately, the C9orf72 orthologue in mice does not produce protein isoforms with different sub-cellular localizations (Atkinson et al., 2015), thus mice would not mimic all aspects of C9orf72 pathology.

Again, as was the case for protein degradation pathways, we see a possible convergence of the mechanisms linked to the C9orf72 expansion (RNA foci, c9DPRs and loss-of-function) in the predicted alterations of nucleocytoplasmic transport.

### **3.2. RNA metabolism**

RNA metabolism is a major aspect of both FTD and ALS. As illustrated in Chapter I, TDP43 has a major role in RNA regulation, splicing, stress granule formation, among others. Aggregated TDP43 may no longer be able to execute normal functions leading to alterations in RNA metabolism. Mutations in RNA-binding proteins TDP43 and FUS, as well as the presence of RNA foci in patients with the C9orf72 mutation, lead to focus attention on RNA toxicity as probably the leading disease mechanism. RNA foci in C9orf72 cases come from the ability of the HRE to form G-quadruplex structures that aggregate in stable forms (Haeusler et al., 2014), leading to the possible capture of RBPs or the cluttering of the RNA processing machinery. Data from patients showed that splicing defects are ~10 times more common than alterations in gene expression level in C9orf72 cases (Anquetil et al. *in preparation*) (Prudencio et al., 2015). Furthermore, RNA foci and c9DPRs seem to induce nucleolar stress (Chapter III) (Kwon et al., 2014; O'Rourke et al., 2015), further altering RNA metabolism. From this point of view, one might think that RNA metabolism and C9orf72

loss-of-function mechanism are completely unrelated, but before reaching a conclusion, it would be interesting to consider some other hypothesis. Loss of C9orf72 function could influence RNA metabolism by altering nucleocytoplasmic shuttling (*refer to nucleocytoplasmic shuttling*) through decreased levels of the short isoform or through nuclear clearance or mislocalization of TDP43. In the first case scenario, as RNA has to transit from nucleus-to-cytoplasm, altered nucleocytoplasmic shuttling would lead to general alterations of RNA profiles in a given cell, including altered TDP43 localization. Then, it could be that this TDP43 mislocalization adds up to deficits in RNA metabolism leading to an altered splicing and transcriptomic profile.

### 3.3. Prion-like spreading and TDP43

One of the main questions in neurodegenerative diseases relates to cellular selectivity, and why some populations are affected rather than others. For example, what will drive upper or lower motor neuron degeneration in ALS, or VEN neurons sensitivity in FTD is a question asked by both clinicians and researchers. Several hypotheses have been raised implicating differences in motor neuron types, the role of axonal transport, and electrophysiological signal of cellular populations, among others (Manuel and Zytnicki, 2011). Anatomically, both motor neurons and VEN neurons are composed of large somas and have long projecting axons marking a possible preferential sensitive. Although the initial triggering event of disease remains elusive, research from the past 2 years suggest that ALS, FTD, and probably other neurodegenerative diseases, might potentially result from a contiguous spread of pathological changes, affecting one section after the other of nearby anatomical regions in a prion-like manner (**Figure 21**) (Ahmed et al., 2016; Burrell et al., 2016; Smethurst et al., 2015). Evidence shows propagation of TDP43 through exosomes (Nonaka et al., 2013) bearing resemblance to the spread of neuronal degeneration (Burrell et al., 2016). And both the presence of a prion-like domain and its seeding properties (Lagier-Tourenne et al., 2010; Smethurst et al., 2015) makes TDP43 the main candidate for this prion-like spreading in ALS and FTD. Exosomes usually fusion directly with the plasma membrane to release components or they can be up-taken via endocytosis. TDP43 propagation may also occur via the release of 'naked' seeds, through nanotubes or via active transport through axons to the synapse. Nearby cells could take up these seeds through various protein-dependent uptake mechanisms (Smethurst et al., 2015). This particular type of spreading would be interesting to explore *in vivo* to see if seeding and propagation is linked to neurotoxicity. A possible paradigm could

be to inject aggregated TDP43 extracts in mice frontal and motor cortex to see if this propagation occurs. It would be interesting to compare this effect to the effect of similar injections in the miR-C9orf72 mouse model, to see if spreading occurs faster or if major local neuronal death takes place.

## Conclusion

This work has allowed to shed light on mechanisms involved in c9FTD/ALS pathology. Altogether, this characterization of a new mouse model of C9orf72 partial loss-of-function showed that C9orf72 alone is not enough to trigger neurodegeneration, but that it is a key component, along RNA toxicity, DPRs aggregation, TDP43 pathology and possibly also aging, of the mechanisms that will trigger and/or drive evolution of the pathology. As C9orf72 seems to have a primordial role in the immunological response, even a partial decrease in expression levels could affect microglial function, and this seems to be due, in part, to altered protein degradation pathways. The miR-C9orf72 mice will be a useful tool for unraveling disease mechanisms and will be likely to provide prime targets for disease-modifying drugs.

Promising gene therapies in mice models using viral delivering of antisense oligonucleotide (ASOs) or RNA interference have been proposed as a possible therapeutic strategy in c9FTD/ALS, but also in TDP43 and SOD1 familial cases. CRISPR technology can also be exploited to target the HRE expansion in a specific manner. Use of antibodies is another potential avenue, since they can target misfolded proteins to reduce the amount of toxic aggregates, or limit disease propagation. Identifying key regions of TDP43 responsible for seeding will allow the development of specific antibody-targeted therapies to halt the propagation. Gene delivery through the use of virus to provide mRNA or cDNA delivery could be useful in the case of loss-of-function mutations. Finally, therapies targeting G-quadruplex and RNA foci formation could be initially tested in models to determine their range of action.

However, no animal model fully replicates the variety of symptoms observed in the human disease, and existing models present significant caveats, thereby limiting translation of preclinical results to clinical studies in humans. The complexity of both FTD and ALS, but also of many other neurodegenerative diseases with large genetic and phenotypic

heterogeneities, makes them particularly hard to model. Mouse models develop additional phenotypes that are not related to the human pathology or model only a particular aspect of the disease. In addition, the high rate of failed translation from preclinical models to clinics (Jucker, 2010), raises concern in the validity of the models for predicting efficacy. The use of mouse models bearing multiple transgenes may model the human pathology more faithfully, but interpretation of the various transgenes and associated lesions may prove quite challenging.

Incomplete mouse models may be advantageous to study specific diseases processes, such as was the case for cell-type specific expression of mutant SOD1 in disease progression (Boillée et al., 2006b). SOD1-induced toxicity in glial cells acts in disease progression, whereas mutant SOD1 in neurons drives onset and initial progression. These models may also be useful for the identification of specific biomarkers linked to a particular alteration, allowing matching between a pathomechanisms and a subsequent underlying cellular pathway. Specific treatments could then be applied to sub-groups of patients that present the previously identified biomarkers to provide a personalized medicine.

As research advances, new mechanisms implicated in neurodegeneration keep emerging. Transcriptomics and proteomics analysis will allow to uncover related pathological pathways, but also to identify specific cellular selectivity, and why some populations are affected rather than others. Models, such as the miR-C9orf72 mice that I characterized during my PhD, can now be further analyzed to understand cell-specific effects of C9ORF72 deficiency, leading to a better understanding of disease mechanisms that will eventually lead to improved patient care and treatment.



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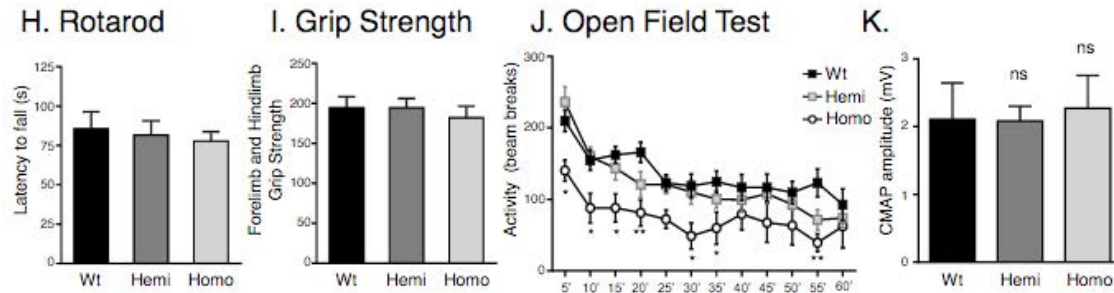
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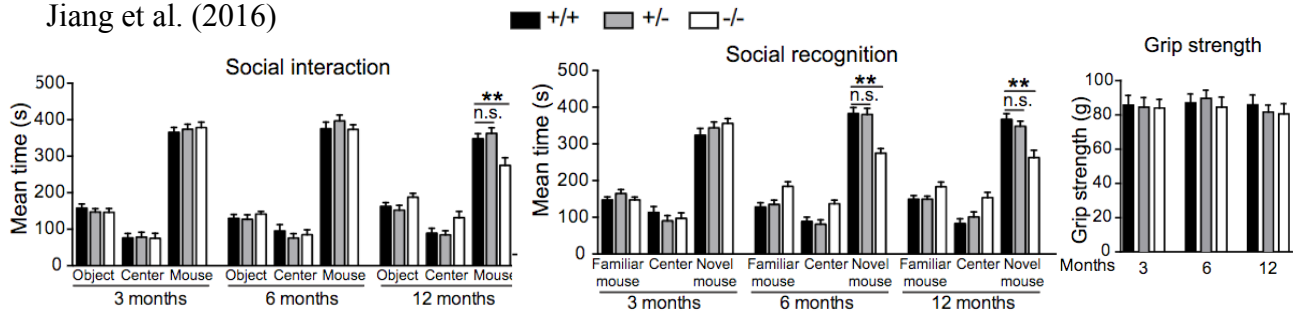
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# ANNEXES

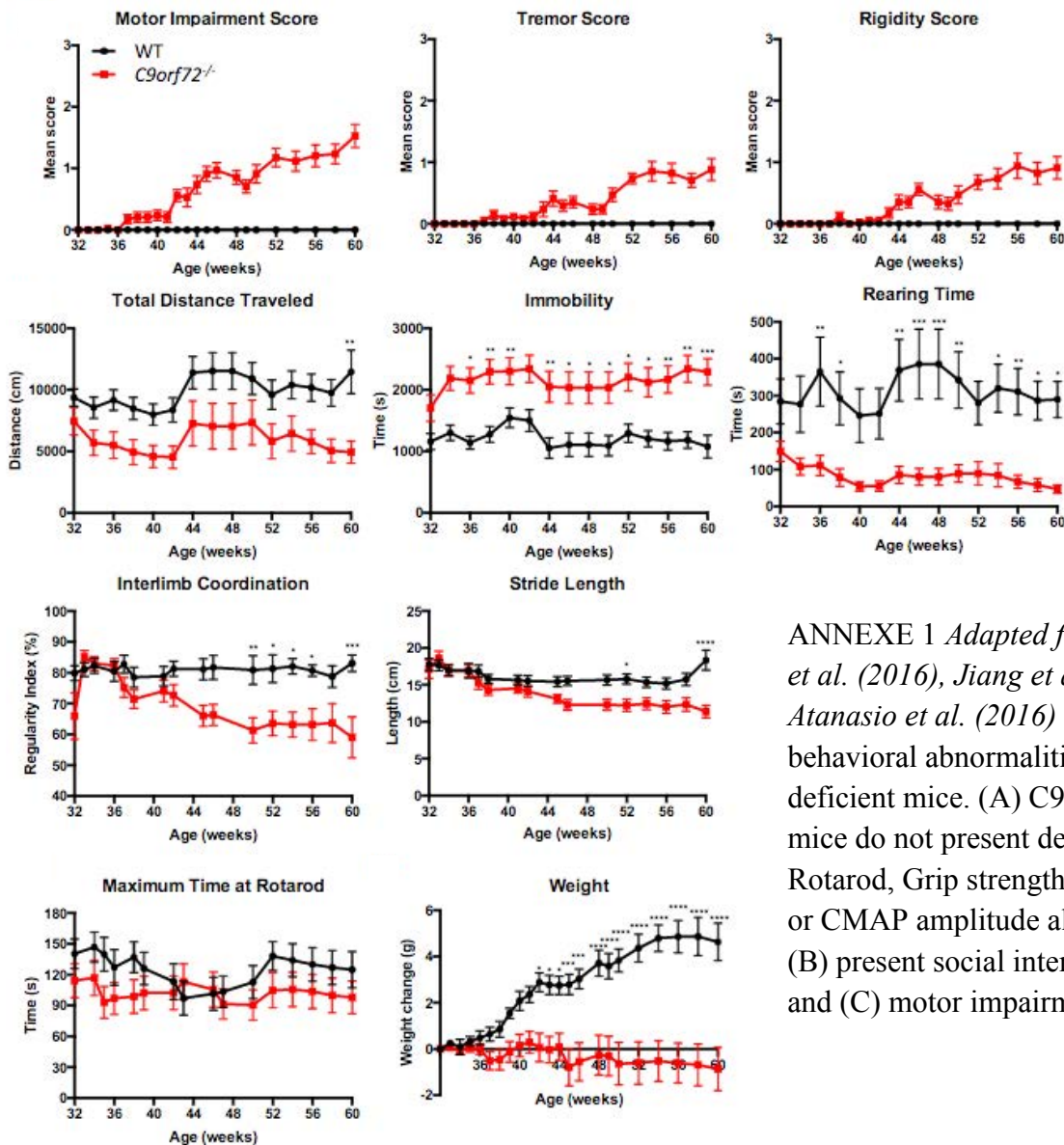
O'Rourke et al. (2016)



Jiang et al. (2016)



Atanasio et al. (2016)



ANNEXE 1 Adapted from O'Rourke et al. (2016), Jiang et al. (2016) and Atanasio et al. (2016) Comparison of behavioral abnormalities of C9orf72 deficient mice. (A) C9orf72 knockout mice do not present deficits in Rotarod, Grip strength or Open Field, or CMAP amplitude alterations but (B) present social interaction deficits and (C) motor impairment

## ABSTRACT

The GGGGCC intronic repeat expansion within *C9ORF72* is the most common genetic cause of amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD). The precise mechanisms through which the *C9ORF72* mutation causes “c9FTD/ALS” have not yet been elucidated, but several hypotheses have been proposed. The mutation may cause a toxic gain of function either through transcription of sense and antisense expanded RNA that aggregates and captures RNA-binding proteins; or through the translation of small aggregating proteins that are produced by repeat-associated non-ATG (RAN) translation. Both the RNA foci and the c9RAN proteins are present in brains of patients carrying the *C9ORF72* mutation and are suspected to cause or participate in the neurotoxicity. Furthermore, the loss of one to three of alternatively spliced *C9ORF72* transcripts and a decreased protein level have been observed in the frontal cortex of patients, implying a concomitant loss of function mechanism.

For my PhD project, in order to investigate the role of this partial loss of function in ALS and FTD disease pathogenesis, we have generated *C9orf72* knock-down mice by targeting the RNA mouse orthologue of *C9ORF72*. We found that by knocking down *C9orf72* intrinsically, mice develop social interaction deficits and depression like behavior, which relate to FTD-like anomalies. When looking for ALS-like abnormalities, we found that *C9ORF72* knock-down mice have conserved spatial memory and normal motility through all their lifespan. However, they develop a lessening of strength that appears late and is maintained without aggravation. They do not present axonal loss or signs of muscle atrophy and/or wasting. Further analysis showed that *C9orf72* knock-down mice present neuromuscular junction deficits at an old age that aggravate with further aging. These findings suggest that *C9ORF72* partial loss of function leads disease pathogenicity course to a more likely FTD-like phenotype, and that gain of function mechanisms as RNA foci and/or c9RAN proteins may be needed to trigger motor neuron disease and massive neurodegeneration.