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INVESTIGATING THE ROLE OF C9ORF72 IN AMYOTROPHIC LATERAL SCLEROSIS USING A ZEBRAFISH MODEL

By

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RÉSUMÉ

La sclérose latérale amyotrophique (SLA) est une maladie neurodégénérative qui affecte les neurones moteurs causant une atrophie musculaire, une paralysie progressive et la mort des patients. À ce jour, il n'existe encore aucun traitement curatif pour la maladie. C'est pourquoi comprendre les mécanismes physiopathologiques de la maladie aidera à identifier de nouvelles cibles thérapeutiques. En 2011, une expansion d'hexanucléotide (GGGGCC) dans la première région intronique du gène *C9orf72* a été découverte comme la cause génétique la plus fréquente de SLA. Cette expansion de répétitions d'hexanucléotide mène à trois mécanismes : deux gains de fonctions toxiques avec une production d'ARN qui vont s'agréger dans les noyaux cellulaires ainsi qu'avec la traduction de ces ARN qui amène à une agrégation de dipeptides dans les cytoplasmes. Le troisième mécanisme est le moins étudié et est un mécanisme de perte de fonction menant à une haploinsuffisance du gène *C9orf72*. Cette perte d'expression tant au niveau de l'ARN que protéique a été rapporté dans des tissus post-mortem de patients. Pour étudier la perte de fonction de ce gène dans la maladie, nous avons utilisé des microARN synthétiques afin de réduire spécifiquement l'expression du gène *C9orf72* chez le poisson zèbre (C9-miARN). Nous avons développé une lignée stable de poisson zèbre C9-miRNA chez laquelle l'expression de *C9orf72* est réduite. En conséquence de la perte de fonction de *C9orf72*, nous avons observé que notre lignée mutante présentait des déficits moteurs sévères à partir de 6 jours post-fertilisation (jpf) et que la majorité des poissons zèbres mouraient prématurément à 15 jpf. Une analyse de la jonction neuromusculaire (JNM) à l'aide de marqueurs pré- et post-synaptiques spécifiques, SV2 et l'alpha-bungarotoxine respectivement, a montré une diminution significative du nombre de JNM chez les C9-miARN à 6 jpf, ce qui corrèle avec une diminution du renouvellement des vésicules synaptiques. La diminution d'expression de SV2a a été confirmé lors d'une analyse en spectrométrie de masse et nous avons également montré une interaction entre C9orf72 et SV2a à l'aide de co-immunoprécipitation, ce qui suggère un fort lien de régulation entre C9orf72 et SV2a. Des enregistrements électrophysiologiques à l'aide de la technique de patch clamp sur des fibres musculaires ont montré une diminution de l'amplitude et de la fréquence des courants de plaques miniatures, ce qui suggère une diminution du nombre de boutons pré-synaptiques. Aussi, nous avons trouvé des agrégats protéiques de TDP-43 chez nos mutants C9-miARN. Parmi les poissons qui ont survécu jusqu'à l'âge adulte, nous avons observé une atrophie sévère des neurones moteurs ainsi que de la moelle épinière. Notre analyse de spectrométrie de masse a également montré une diminution de l'expression de la calpastatine

dans notre lignée mutante, une protéine neuroprotectrice. Nous avons donc pharmacologiquement compensé cette perte d'expression de la calpastatine et observé un sauvetage phénotypique partiel du nombre de JNM ainsi que du renouvellement des vésicules synaptiques, ce qui indique que cette voie pourrait être dérégulée et mener à la dysfonction synaptique observée. Ensemble, ces résultats montrent que notre lignée C9-miARN représente un bon modèle de la SLA, démontrent un rôle de C9orf72 pour la transmission synaptique à la JNM et enfin, notre modèle ouvre la voie pour étudier l'effet neuroprotecteur de la calpastatine en tant que potentiel traitement thérapeutique.

Mots-clés : Sclérose latérale amyotrophique, maladie neurodégénérative, poisson zèbre, C9orf72, neurone moteur, jonction neuromusculaire, calpastatine.

ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease that affects motoneurons causing muscular atrophy, paralysis and ultimately death. Presently, no curative treatment exists. Understanding the physiopathological mechanisms can help identify new therapeutic targets. In 2011, an expansion of a repetition of a hexanucleotide (GGGGCC) in the first intronic region of the *C9orf72* gene has been discovered as the first genetic cause of ALS. This expansion of hexanucleotide repetition leads to three pathological mechanisms: two toxic gain of function with the production of RNA aggregating mainly in the cell nucleus and with the translation of these RNAs leading to dipeptide repeats aggregating in the cell cytoplasm. The third mechanism is the less studied and is a loss of function of the gene *C9orf72* leading to its haploinsufficiency. This loss of expression both at the RNA and protein levels has been reported from post-mortem patient tissue. To investigate the role of *C9orf72* loss of function in ALS, we used four synthetic micro-RNAs to specifically target the zebrafish *C9orf72* gene (C9-miRNA) and have developed a stable zebrafish C9-miRNA line with reduced expression of *C9orf72*. Upon loss of function of *C9orf72*, we observed that zebrafish C9-miRNA mutants display severe motor deficits beginning 6 days postfertilization (6 dpf) and a majority die prematurely at 15 dpf. Analysis of the neuromuscular junctions using specific presynaptic and postsynaptic markers SV2a and alpha-bungarotoxin respectively, revealed a significant decrease in the number of synaptic contacts in the C9-miRNA line at 6 dpf correlating with a decreased synaptic vesicles turnover. SV2a loss of expression was confirmed in a mass spectrometry analysis that showed a great reduction of SV2a protein level, and we showed that C9orf72 interacts with SV2a using Co-IP technique strongly suggesting a regulation between C9orf72 and SV2a. Electrophysiology recordings using patch clamp technique on muscle fibres showed a decrease of amplitude and frequency of the spontaneous miniature end plate currents, which suggests a decreased number of presynaptic endings. Also, TDP-43 has been shown to aggregates starting 6 dpf in our C9-miRNA. Among the few fish that survived until adulthood, we observed a significant motoneuron and muscle atrophy. Mass spectrometry also showed a decreased expression of the neuroprotective protein, calpastatin in our mutant. Thus, we pharmacologically compensated the loss of calpastatin and observed some rescue of the number of synaptic contacts and vesicle turnover indicating that this pathway may have a role in the synaptic dysfunction. Altogether, our zebrafish C9-miRNA replicates aspects of ALS and showed that C9orf72 has a role in the synaptic transmission at the NMJ, last, the model brought new avenue to study neuroprotection by working on the calpastatin pathway.

Keywords: Amyotrophic lateral sclerosis, neurodegenerative disease, zebrafish, C9orf72, motoneuron, neuromuscular junction, calpastatin.

SOMMAIRE RÉCAPITULATIF

La sclérose latérale amyotrophique (SLA) est une maladie neurodégénérative qui mène à la dégénérescence des neurones moteurs, les neurones impliqués dans le mouvement. Suite à cette perte, les patients montrent une faiblesse musculaire menant à une paralysie progressive et à la mort, le plus souvent dans les cinq années suivant le diagnostic. Encore à ce jour, aucun traitement curatif n'existe. Seulement deux traitements sont disponibles pour les patients, l'Edaravone et le Riluzole, mais ces deux molécules n'ont que des effets minimes sur le ralentissement de la progression de la maladie et sont accompagnés d'effets secondaires majeurs. Les mécanismes menant à la SLA sont variés, nombreux et très étudiés afin de trouver un traitement plus efficace. Près d'un millier d'essais cliniques sont en cours afin de tester de nouveaux traitements pour la maladie (Tableau 1.1).

La SLA a été liée à plusieurs causes génétiques et environnementales. Dans ce projet, nous nous intéressons à la cause génétique la plus commune, le gène *C9orf72*. La mutation trouvée en 2011 dans ce gène est une expansion d'un héxanucléotide GGGGCC dans la première partie intronique du gène. Elle est responsable d'environ 40% des cas familiaux de SLA et représente environ 8% des cas sporadiques. Lorsque cette mutation a été identifiée, le rôle de *C9orf72* étaient encore inconnu. La mutation retrouvée chez les patients engendre trois différents mécanismes : deux gains de fonction toxiques avec la transcription de la répétition d'héxanucléotide générant des ARN qui s'accumulent en foci dans les noyaux cellulaires, la traduction de ces ARN via la voie non canonique de traduction non dépendante d'un codon AUG mais liée à des répétitions menant à la production de dipeptides qui s'agrègent dans le cytoplasme principalement. Ces deux gains de fonction toxiques sont très étudiés afin d'identifier des moyens de les éviter. Mais le troisième mécanisme induit par la mutation est une diminution de l'expression du gène *C9orf72*. En effet, les tissus des patients ont montré un niveau plus faible de transcrits de *C9orf72*. Ce dernier mécanisme est le moins étudié, pourtant comprendre l'impact de cette baisse d'expression peut mener à de potentielles voies thérapeutiques également. Depuis 2011, C9orf72 a été largement étudié et a été retrouvé comme impliqué dans divers processus cellulaires tels que l'autophagie, la croissance axonale ou la régulation de la réaction immunitaire puisque ses transcrits ont été retrouvés dans des cellules myéloïdes chez des patients. Le rôle le plus étudié de C9orf72 est dans l'autophagie puisque sa séquence protéique montre la présence d'un domaine différentiellement exprimé dans les tissus normaux et néoplasiques (DENN) qui est connu pour être un facteur d'échange de guanine (GEF) pour les

rabs. Les rabs étant impliqués dans les processus vésiculaires dans la cellule. C9orf72 a également été localisé dans différents compartiments cellulaires dont le compartiment synaptique, mais son rôle à cet endroit demeure inconnu. C'est pourquoi notre projet porte sur l'identification du rôle de C9orf72 à la synapse et pour se faire, nous avons étudié la synapse la plus impactée dans la SLA : la jonction neuromusculaire (JNM). Notre hypothèse étant que la perte d'expression de C9orf72 engendre des défauts synaptiques majeurs menant à la perte de la JNM et donc à la paralysie.

Pour se faire, nous avons généré un modèle de poisson zèbre *Danio rerio*, un modèle vertébré ayant 76% d'homologie pour le gène C9orf72, chez lequel nous avons intégré de façon stable quatre micro-ARN synthétiques dans la séquence 3' UTR de l'orthologue de C9orf72. Cette intégration a permis de diminuer l'expression du gène de façon significative ainsi que l'expression protéique de 50%. La stabilité de cette expression nous a permis de faire grandir nos poissons afin d'étudier l'effet de cette baisse d'expression au stade adulte.

Les premières observations que nous avons pu faire dans notre modèle a été une altération de la morphologie à partir de 10 jours post fertilisation (jpf), accompagné d'une mort précoce à 15 jpf. Nous avons observé une diminution significative de l'activité motrice à partir de 6 jpf qui a été lié à une perte du nombre de JNM. Ces JNM ont été imagé à l'aide de deux marqueurs : SV2 et l'α-bungarotoxine, des marqueurs pré- et post-synaptiques respectivement. Nos poissons adultes ont également montré une forte diminution de l'activité motrice ainsi qu'une atrophie de la moelle épinières, des neurones moteurs et des muscles. De façon inattendue, nous avons également rapporté le fait que la protéine TDP-43, agrégée dans près de 99% des cas de SLA, l'était aussi dans notre modèle. Ce qui suggère une régulation entre C9orf72 et TDP-43.

Au niveau synaptique, nous avons évalué le recyclage des vésicules synaptiques et nous avons constaté une diminution significative de ce recyclage au niveau des JNM chez nos mutants. Cette baisse est corrélée avec diminution significative de la fréquence et de l'amplitude des courants de plaques miniatures obtenus grâce à des enregistrements en électrophysiologie. Enfin, nous avons évalué le niveau d'expression et la localisation de vésicules positives pour Rab3a à la JNM et nous avons également observé une diminution drastique du nombre de puncta positifs pour Rab3a ainsi qu'une diminution de l'aire de ces puncta.

Finalement, une étude de spectrométrie de masse nous a donné deux résultats à exploiter : notre marqueur pré-synaptique SV2 est la première protéine dont l'expression est diminuée chez nos mutants. Cette donnée a ensuite été corroborée par une expérience de co-immunoprécipitation qui montrait une interaction entre C9orf72 et SV2, pouvant expliquer la régulation entre ces

protéines.

La seconde donnée extraite et utilisée ici a été que la calpastatine est la seconde protéine la plus négativement régulée dans notre modèle. La calpastatine est l'inhibiteur de la calpaïne qui est une enzyme clivante activée par une augmentation du calcium intracellulaire. Une lecture approfondie à propos de cette protéine nous a indiqué qu'elle a un effet neuroprotecteur et qu'une baisse de son expression avait déjà été rapporté dans d'autres modèles de SLA ainsi que dans d'autres maladies neurodégénératives telles que la maladie de Parkinson, d'Alzheimer ou de Huntington. Nous avons donc traité nos mutants *C9orf72* avec deux molécules : un inhibiteur de la calpaïne et un peptide de 27 acides aminés représentant le site actif de la calpastatine. Ces deux traitements ont montré des effets bénéfiques sur la fonction motrice de nos mutant avec une augmentation du nombre de JNM. Une augmentation du recyclage des vésicules synaptiques à la JNM a également été observée. Enfin, une expérience d'immunomarquage nous a permis de visualiser la calpastatine dans des tissus de cerveaux de patients. Ce que nous avons observé est une localisation nucléaire chez les patients alors que la localisation est cytoplasmique majoritairement chez nos contrôles. Cette délocalisation serait expliquée par le fait que lorsque la calpastatine est inactive, elle est localisée au noyau, contrairement à lorsqu'elle est activée et donc exportée au cytoplasme. Ceci indiquerait que les patients ont bel et bien un défaut au niveau de la voie calpaïne/calpastatine.

En conclusion, nous avons généré et caractérisé un nouveau modèle de la SLA, un poisson zèbre pour lequel nous avons diminué l'expression de *C9orf72*, ce modèle a montré les caractéristiques attendues pour l'étude de la SLA incluant les défauts moteurs et l'agrégation de TDP-43. Nous avons appris que C9orf72 a un rôle à la JNM et qu'une perte de son expression engendre des défauts sévères menant à la perte de la JNM. Nous avons également testé deux molécules comme potentiels traitements pour complémenter la perte d'expression de la calpastatine dans notre modèle.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

AAV: Adeno-associated virus

ALS: Amyotrophic lateral sclerosis

AMO: Antisense morpholinos

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

ARF: ADP ribosylation factor

ASO: Antisense oligonucleotide

ATP: Adenosine triphosphate

BAC: Bacterial artificial chromosome

C9orf72: Chromosome 9 open reading frame 72

CNS: Central nervous system

CRISPR: Cluster regularly interspaced short palindromic repeats

DENN: Differentially expressed in normal and neoplasia

DNA: Desoxyribonucleic acid

dpf: days post fertilization

DPR: Dipeptide repeat

DPS: Diaphragm pacing system

fALS: familial amyotrophic lateral sclerosis

FDA: Food and drug administration

FTD: Fronto-temporal dementia

FISH: Fluorescent in situ hybridization

GA: Glycine - alanine

GABA: γ-aminobutyric acid

GAP: GTPase activating protein

GDP: Guanosine diphosphate

GEF: Guanine exchanging factor

GFP: Green fluorescent protein

GOF: Gain of function

GP: Glycine – proline

GR: Glycine - arginine

GTP: Guanosine triphosphate

GWAS: Genome wide association study

hnRNP: heterogenous nuclear ribonucleoprotein

hpf: hours post-fertilization

HRE: Hexanucleotide repeat expansion

iMN: induced motoneuron

iPSc: induced pluripotent stem cell

LIR: LC3 interacting region

LMN: Lower motoneuron

LOF: Loss of function

mEPC: miniature endplate current

miRNA: microRNA

NCT: Nucleo-cytoplasmic transport

NES: Nuclear export sequence

NLS: Nuclear localization sequence

NMJ: Neuromuscular junction

NPC: Nuclear pore complex

PA: Proline - alanine

PR: Proline - arginine

RAN: Repeat-associated non-ATG

RBP: RNA binding protein

RNA: Ribonucleic acid

RNAseq: RNA sequencing

ROS: Reactive oxygen species

RRM: RNA recognition motif

sALS: sporadic amyotrophic lateral sclerosis

SNARE: Soluble N-ethylmaleimide-sensitive factor attachment

tSMS: transcranial static magnetic stimulation

UBA: Ubiquitin associated domain

UMN: Upper motoneuron

UTR: Untranslated region

VGCC: Voltage gate calcium channel

WES: Whole exome sequencing

WGS: Whole genome sequencing

1 INTRODUCTION

1.1 AMYOTROPHIC LATERAL SCLEROSIS

1.1.1 Clinical features

Amyotrophic lateral sclerosis (ALS) is a rare neurodegenerative disease that affects motoneurons leading to a progressive muscle weakness, muscle atrophy, fasciculations, muscle cramps, slowness of movement and muscle stiffness. Symptoms of the disease have first been described in 1869 by a French neurologist, Jean-Martin Charcot. This is a fatal disease and patients die within 2 to 5 years after the diagnosis most frequently due to a respiratory failure (Chio *et al.*, 2009; Turner *et al.*, 2009). These symptoms are due to a progressive degeneration of upper motoneurons (UMNs), projecting form the cortex to the brainstem or the spinal cord and lower motoneurons (LMNs), projecting from the brainstem or the spinal cord to the muscles. After the loss of the neuromuscular connection, an axonal retraction is observed and UMNs and LMNs die.

Because of its various forms of initial presentation of the disease, age of onset, progression rate and survival, ALS is a highly heterogenous disease that make the diagnosis difficult to establish (Kiernan *et al.*, 2011). The heterogeneity is partly explained since ALS has two clinical forms, bulbar and spinal. Bulbar onset leads to difficulty in swallowing (dysphagia) and speaking (dysarthria), hyperreflexia, spasticity and pseudo bulbar affect resulting from the degeneration of the corticobulbar neurons in the brainstem. It represents 25 to 30 percent of ALS cases and the progression is faster than in the spinal form. Spinal onset leads to limb weakness, atrophy, fasciculations, hyporeflexia and paralysis resulting from the death of spinal motoneuron. This form represents 70 to 75 percent of ALS cases.

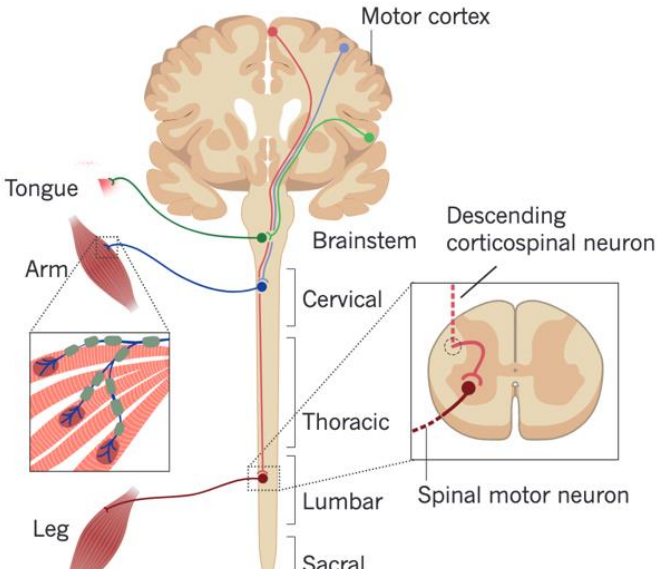

Figure 1-1: ALS affected system

ALS affects corticospinal motoneurons that project the cortex to the brainstem or the spinal cord and the bulbar and spinal motoneurons that project to the muscles, from the adapted from (Taylor *et al.*, 2016). Rights obtained from the copyright clearance center and Springer Nature (License: 5312531087212)

ALS has several epidemiological causes. Around 5 to 10 percent of the cases are familial (fALS) and have a genetic cause but most of the cases are sporadic (sALS) (Talbott *et al.*, 2016). These sALS can be explained by genetic causes as well as environmental factors such as aging, smoking or exposure to toxins. ALS affects around 0.05 percent of the population or 4 to 6 persons per 100 000. The sex ratio is 1.5 males for 1 female. In Canada, ALS is the neurodegenerative disease that causes to more deaths.

ALS is in a spectrum disorder with fronto-temporal dementia (FTD). 5 to 15 percent of ALS patients have FTD and up to 50 percent of them shown FTD related cognitive impairment (Kiernan *et al.*, 2011). Similarly to ALS, FTD is a progressive neurodegenerative disease however FTD affects neurons from the frontal and temporal lobes. Clinically, FTD patients show changes in behavior, language function and personality and 30 percent of FTD patients develop ALS. FTD was first described in 1892 by Arnold Pick and is now considered as the second most common early-onset dementia (Harvey *et al.*, 2003). These two diseases were initially considered as independent (Woollacott *et al.*, 2016) but discovering C9orf72 in 2011created a bridge between ALS and FTD. Since then, 11 genes have been identified as susceptibility genes for both ALS and FTD.

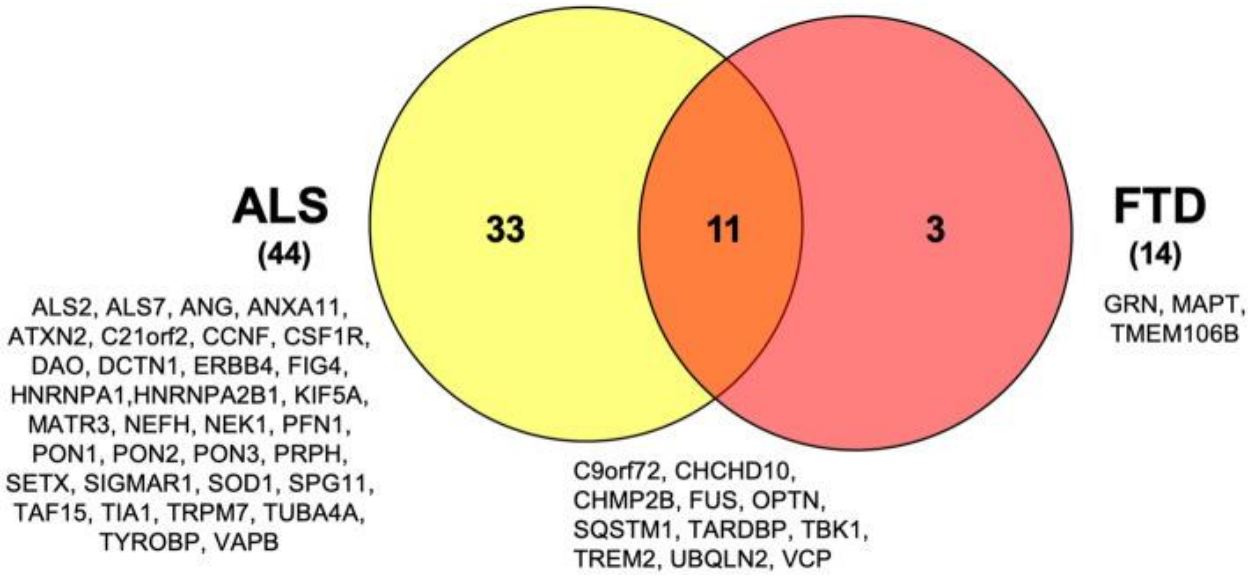

Figure 1-2: ALS-FTD spectrum genetic overlap.

More than 50 genes have been identified as causative in ALS and FTD, this diagram shows the different genes and their overlapping between the two diseases. Adapted from (Liscic *et al.*, 2020) (Authorized by CC BY 4.0 license, Creative commons).

1.1.2 Diagnosis of ALS

Because of the high heterogeneity in the clinical features observed in patients, ALS is tough to diagnose. The diagnosis can be done using different tools such as the El Escorial score, the Gold Coast score or even the ALS Functional rating scale score (ALS FRS) (Brooks, 1994; Brooks *et al.*, 2000; Kollewe *et al.*, 2008; Shefner *et al.*, 2020) .

According to the revised El Escorial, ALS is defined by the presence of UMN and LMN degeneration that can be evaluated by electrophysiological, neuropathological and clinical examination as well as a progressive spread of the symptoms. This score also requires the absence of other evidence of a pathology to explain the neurodegeneration either using electrophysiological or pathological examination, either using imaging techniques such as MRI for example (Brooks *et al.*, 2000).

The Gold Coast score requires a progressive motor impairment as well, assessed several times accompanied by the presence of UMN and LMN dysfunction in at least one body region that can be bulbar, cervical, thoracic or lumbosacral and tests to exclude other disease explaining the symptoms (Shefner *et al.*, 2020).

The ALS FRS is giving information about the severity and the progression of the disease, it is used as a tool during clinical trials as well. It measures functional criteria such as the intelligibility of the speech, the salivation, swallowing, handwriting, walking, climbing stairs, dyspnea, respiratory insufficiency (Kollewe *et al.*, 2008).

Overall, ALS diagnosis remains a diagnosis of exclusion and because of the heterogeneity of the symptoms and even during the early stages of the disease, the diagnosis takes most of the time between 9 and 15 months to be given, from the first symptoms (Al-Chalabi *et al.*, 2013; Chio, 1999).

1.1.3 Available treatments

To this day, no curative treatment is available. Available and approved molecules have very mild effects by only increasing patient’s lifespan over a couple of months.

Riluzole is the first approved molecule for ALS and has a role against excitotoxicity. It inhibits the glutamatergic release at the pre-synaptic terminus. Riluzole increases bulbar and limb functions although it does not have an effect over muscle strength (Bensimon *et al.*, 1994; Miller *et al.*, 1996; Rothstein, 1996).

Edaravone is another approved molecule and has a role on oxidative stress. This treatment is administered by intravenous injection and has cytoprotective and neuroprotective effect against oxidative stress. Edaravone helps to eliminate Reactive Oxygen Species (ROS) that are accumulating and leading to neuronal damages because of the peroxidation of the unsaturated fatty acids in the neuronal cells (Brooks *et al.*, 2018; Rothstein, 2017; Witzel *et al.*, 2022).

Masitinib is a kinase inhibitor that has a role in neuroprotection by targeting immune cells in the nervous system. Its main targets are mast cells and microglia (Trias *et al.*, 2016; Trias *et al.*, 2018). Masitinib is approved by the FDA for other diseases and new studies show an actual benefit from it by extending lifespan of ALS patients. It could also have an effect in synergy with Riluzole by slowing down the functional decline of 27% compared to Riluzole alone (Mora *et al.*, 2020).

Very recently, Albrioza treatment which combines sodium phenylbutyrate and tauroursodeoxycholic acid, was approved in Canada for Phase III after proving it could slow down disease progression (Paganoni *et al.*, 2021; Paganoni *et al.*, 2020). The cellular effect of the treatment is by reducing the endoplasmic stress response and decreasing cell apoptosis (Daruich *et al.*, 2019; Kubota *et al.*, 2006).

Many drugs are currently in clinical trials as potential treatments for ALS. For example, Ibudilast is currently in phase IIb/III and aims to protects neurons from inflammation (Oskarsson *et al.*, 2021), Reldesemtiv which is in phase II trial and aims to improve muscle contractility (Shefner *et al.*, 2021) and GDC-0134 which is in phase I and is supposed to protect from axon degeneration and apoptosis (Katz *et al.*, 2022). The use of simple genetic model as the nematode, Caenorhabditis elegans or the Zebrafish have contributed to the discovering of new effective molecules that are now in clinical trial, such as Pimozide having neuroprotective effect on the NMJ (Patten *et al.*, 2017). Induced pluripotent stem cells (iPS) have also been helpful since a team could perform a drug screen on patients derived neurons and take the Ezogabine in clinical trial (Wainger *et al.*, 2021). In the table 1, we can see that although many clinical trials are ongoing and passing phase 1 and 2, most of these studies fail at phase 3 since the number of patients increases which unravels fake positive. The lack of biomarkers is a major problem to the understanding of the trial failure.

Table 1-1.1: Recapitulative table of ongoing clinical trials for ALS

(Source: NIH)

Drug	Phase	Mechanism of action
ABBV-CLS-7262	Phase 1	Initiation factor EIF2b activator, protects from unfolded proteins toxicity
Bosutinib	Phase 1	Src/c-Abl inhibitor, induces autophagy and reduces misfolding of SOD1 and TDP-43
DNL-343	Phase 1	Initiation factor EIF2b activator, protects from unfolded proteins toxicity
GDC-0134	Phase 1	Dual Leucine Zipper Kinase (DLK) inhibitor, protects form axon degeneration and apoptosis
Trametinib	Phase 1/2a	MEK inhibitor
3K3A-APC	Phase 2	Human activated protein C replacement protein, decreases inflammation
AP-101	Phase 2	Human antibody, binds misfolded form of SOD1 protein
Enoxacin	Phase 2	Dicer activator, involved in miRNA regulation in motoneurons
Ezogabine	Phase 2	Potassium channel activator, decreases motoneuron excitability
Fasudil	Phase 2	Rho kinase inhibitor, increases neurone regeneration and motor functions
Gilenya	Phase 2	Sphingosine 1-phosphate (S1P) receptor inhibitor, prevents inflammation
Ibudilast	Phase 2	Phosphodiesterase (PDE)-4 and -10 inhibitor, prevents inflammation
Inosine	Phase 2	Elevates urate level, protects against oxidative stress
Mexiletine	Phase 2	Sodium channel inhibitor, reduces muscle cramps
Pegcetacoplan	Phase 2	Complement protein 3 (C3) inhibitor, decreases inflammation at the NMJ
Pimozide	Phase 2	T-type calcium channel antagonist, stabilizes the NMJ
Rasagiline	Phase 2	Monoamine oxidase-B inhibitor, increases dopamine levels, neuroprotective
Reldesemtiv	Phase 2	Fast skeletal muscle troponin activator (FSTA), increases skeletal muscle contractility
Sotuletinib	Phase 2	CSF-1R inhibitor, microglia activator
Ceftriaxone	Phase 3	Increases glutamate transporter EAAT2 expression, protects from glutamate -induced toxicity
Dexpramipexole	Phase 3	Dopamine agonist, antioxidant, apoptosis inhibitor, free radical scavenger
Albrioza	Phase 3	Blocker of mitochondria and endoplasmic reticulum stress

Another existing therapy is based on antisense oligonucleotides (ASO) and only targets fALS since it requires a genetic mutation. ASOs are injected and can go through the blood-brain barrier to be available to the Central Nervous System (CNS) including neurons and glial cells. They have shown effect in patients with decrease of the problematic transcripts in their cells (Tran *et al.*, 2022). The main drawback of ASOs is that they are limited to patients with specific gene mutations such as *SOD1* and *C9orf72* so far.

Table 1-2.1: Table of ASOs therapies in trial

(Source: NIH)

ASOs name	Gene targeted	Status of the trial
Tofersen	*SOD1*	Phase 3
Afinersen	*C9ORF72*	-
BIIB078	*C9ORF72*	Failed
WVE-004	*C9ORF72*	Phase 1b/2a
Jacifusen	*FUS*	Phase 3
BIIB105	*ATAXN2*	Phase 1

Lastly, non-drug trials are ongoing. Several involving the diaphragm pacing system (DPS) that uses intramuscular electrodes to stimulates the diaphragm to inflate the lungs and allow normal breathing. Since respiratory failure is what lead to patient death, DPS could be a great tool. transcranial static magnetic stimulation (tSMS) is a non-invasive brain stimulation that decreases motor cortex excitability. This stimulation would have an effect on hyperexcitability seen in ALS patients. It has been tested on two patients and although both patients had a rapid and aggressive form of ALS, the stimulation seems to have given encouraging results leading the study further (Lazzaro *et al.*, 2022).

1.2 Genetics of ALS

Around 5 to 10 percent of the ALS cases are familial (fALS) and have a genetic cause but most of the cases are sporadic (sALS) (Talbott *et al.*, 2016). Linkage analysis on large ALS families in the nineties allowed the identification of *SOD1* in 1993 as the first genetic cause of the disease and with the development of new techniques like genome wide association studies (GWAS), whole exome sequencing (WES) and whole genome sequencing (WGS), more than 40 new genes have been discovered within less than 20 years. They affect a very broad range of cellular function and give various cellular defects yet leading to homogenous clinical phenotype. Table 2 recapitulates identified ALS genes and their main features. *SOD1, TARDBP, FUS* and *C9orf72* are the four main ALS genes involved in around 55 percent of fALS and 10 percent of sALS.

1.2.1 SOD1

Superoxide dismutase 1 (*SOD1*) was the first gene identified as a genetic cause for ALS in 1993 (Rosen *et al.*, 1993). *SOD1* is located on chromosome 21q22.11 and encodes a 154 amino acids long protein. SOD1 is a homo-dimeric metalloprotein which function is antioxidant by dismutating the free superoxide radicals into molecular oxygen and hydrogen peroxide. More than 180 mutations of all kinds, insertions, deletions, single point mutation or even truncation have been reported to lead to protein defects as misfolding and aggregation involved in ALS. Amongst these, two mutations are mostly found: D90A and A4V. Another mutation, G93A, was the first to be used to generate transgenic mouse models that recapitulated ALS phenotypes and is widely studied but remains a relatively rare mutation. *SOD1* patients represent around 15 percent of fALS and around 1 percent of sALS in European population although it is more frequent in Asian population reaching 30 percent of fALS in this population (Mejzini *et al.*, 2019).

SOD1 toxicity has been linked to its increased aggregation, homodimer destabilization and oligomerization in ALS. It has also been seen in sALS patients that the healthy form of SOD1 was toxic through oxidation (Ezzi *et al.*, 2007; Gruzman *et al.*, 2007).

1.2.2 FUS

Fused in sarcoma (*FUS*) is located on chromosome 16p11 and encodes a 526 amino acids long protein. This protein has an RNA recognition motif, a glycine-serine-tyrosine (QGSY) rich, low complexity, prion-like domain in its N-terminus, three arginine-glycine-glycine (RGG) rich motifs, a zinc finger domain and a nuclear localization signal in its C-terminus. It has a role in RNA processes such as RNA splicing, maturation, stability and transport. FUS can also interact with

Histone Deacetylase 1 (HDAC1) and disrupt the DNA damage repair process (Kwiatkowski *et al.*, 2009).

In 2009 *FUS* has been identified as involved in ALS and since then, more than 79 mutations have been reported (Kwiatkowski *et al.*, 2009; Vance *et al.*, 2009) . *FUS* mutations are involved in 4 percent of fALS cases and 1 percent of sALS. Most of the mutations are found in the glycine rich, prion-like domain and the NLS domain. Some mutations in the gene lead to a mislocalization of the protein from its regular nuclear localization to the cytoplasm while other lead to large, ubiquitin-positive, TDP43-negative cytoplasmic inclusions.

1.2.3 TARDBP

TAR DNA Binding Protein (*TARDBP*) is located on chromosome 1p36.22 and encodes a 414 amino acids long DNA/RNA binding protein, TDP-43. TDP-43 contains a N-terminus domain that promotes oligomerization, two RNA recognition motifs (RRM1 and 2) which bind UG-rich sequences, a nuclear localization and export signals as well as a low complexity domain and a glycine rich domain in the C-terminus (Buratti *et al.*, 2001; Wang *et al.*, 2004). The LCD is the domain where most of ALS related mutations are found.

TDP-43 is a member of the heterogenous nuclear ribonucleoprotein (hnRNP) family which plays a role in multiple steps of RNA processing. Most of TDP-43 is found in the nucleus and has a role in RNA splicing. TDP-43 binds to (UG)n consensus binding motifs in mRNA introns and regulates their splicing and stability (Buratti *et al.*, 2001). Amongst the mRNA regulated by TDP-43, many genes encoding proteins involved in neuronal development, axonal guidance and synaptic activity. That can explain how a mutation in TDP-43 leads to synaptic defects. Although, TDP-43 is mainly found in the nucleus, it can be found in the cytoplasm thanks to its export signal, where it is involved in mRNA transport. In 2008, TDP-43 has been found to be associated with ALS as responsible for 5 percent of fALS and 1.5 percent of sALS (Sreedharan *et al.*, 2008). It has also been found aggregated in more than 97 percent of all ALS cases. They key feature in TDP-43 pathology in ALS its mislocalization from the nucleus to the cytoplasm (Neumann *et al.*, 2006). Indeed, since 2006, TDP-43 was identified is a major component of ubiquitin positive inclusions found in brain tissues from ALS patients and only *SOD1* and *FUS* patients do not seem to show TDP-43 positive aggregates (Mackenzie *et al.*, 2007).

TDP-43 mutations give both a gain and a loss of function mechanisms. Gain of function (GOF) mechanism leads to its toxic cytoplasmic aggregation. As a GOF, it has been seen that increased level of TDP-43 transcripts levels correlate with impaired cognitive functions although no sign of

motoneuron degeneration was observed. Loss of function (LOF) mechanism leads to splicing defects. A study shows that 885 genes are affected in motoneurons. Stathmin-2 (STMN2) is a gene expressed in differentiated neurons and more specially in the growth cone during axon extension and repair (Klim *et al.*, 2019). *STMN2* expression is regulated by TDP-43 therefore a mutation in this gene leads to the appearance of a cryptic exon giving an abnormal isoform of STMN2 and a decrease of expression of its normal isoform affecting the axonal outgrowth and repair.

1.2.4 C9orf72

Chromosome 9 Open Reading Frame 72 (*C9ORF72)* mutation has been discovered in 2011 in ALS patients. The mutation found in this gene is a Hexanucleotide Repeat Expansion (HRE): GGGGCC, in the first intronic pat of the gene. *C9ORF72* has 11 exons and is located on chromosome 9p21 (DeJesus-Hernandez *et al.*, 2011; Renton *et al.*, 2011). The first exon is a non-coding exon and can be 1a or 1b depending on the transcript. Indeed, *C9ORF72* in human has 3 transcripts: V1, V2 and V3. V1 and V3 include the first exon as 1a whereas V2 include the first exon 1b. V1 is the short isoform of the protein and is 24kDa. V2 and V3 are the long isoforms and are 54kDa. V2 is the most expressed isoform and in ALS, V1 and V3 can be the problematic transcripts since the HRE is found between 1a and 1b parts of the gene.

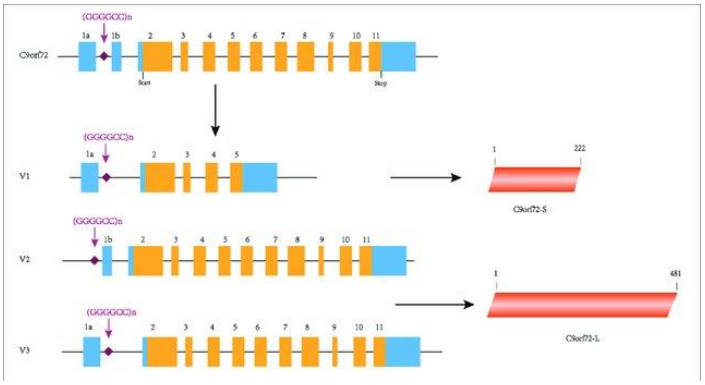

Figure 1-3: Schematic representation of C9orf72 different transcripts.

Source: (Yang *et al.*, 2020). (Authorized by CC BY 4.0 license, Creative commons).

C9ORF72 is the most common genetic cause of ALS since it represents almost half of fALS cases and around 5 to 10 percent of sALS (Ghasemi *et al.*, 2018). The HRE threshold has been arbitrary set to 30 repetitions of the GGGGCC motif to be pathologic. This HRE leads to three pathological mechanisms, two toxic GOF and a LOF mechanism. The first toxic GOF is the accumulation and aggregation of RNA foci in the cell nuclei. Indeed, the transcription of exon 1a leads to the production of sense and antisense RNA foci that have been detected by fluorescent in situ hybridization (FISH) (Cooper-Knock *et al.*, 2014; Donnelly *et al.*, 2013; Mizielinska *et al.*, 2013). The second toxic GOF is due to the non-canonical way, repeat-associated non-ATG (RAN)

translation leading to the production of five different dipeptides aggregating in cell cytoplasm (Ash *et al.*, 2013; Gendron *et al.*, 2013; Mackenzie *et al.*, 2013; Mackenzie *et al.*, 2014; Zu *et al.*, 2013). The third mechanism is the LOF of the endogenous *C9ORF72* leading to haploinsufficiency (DeJesus-Hernandez *et al.*, 2011; Donnelly *et al.*, 2013; Gijselinck *et al.*, 2012). The HRE being in the promoter region, it prevents transcript V2 from being transcribed and it results in an overall decrease of *C9ORF72* level of transcripts. These three pathological mechanisms will be detailed further down (Section 1.6). Post-mortem tissues have been showing both RNA foci, dipeptide repeats and TDP-43 positive inclusions. Decreased levels of *C9ORF72* transcripts have been reported in those tissues as well. These hallmarks are key features of *C9ORF72* pathology (DeJesus-Hernandez *et al.*, 2011).

C9ORF72 mutant motoneurons are sensitive to glutamate toxicity and a study shows that a an ASO targeting the HRE decreases this sensitivity. This is explained by the fact that RNA foci would bind ADARB2 protein (Donnelly *et al.*, 2013). This protein is usually involved in the edition of GluR2 α-amino-3-hydroxy-5-methyl-4isoxazolepropionic acid (AMPA) receptor and increases calcium impermeability (Kawahara *et al.*, 2003a; Kawahara *et al.*, 2003b). That is why, binding ADARB2 sequestration increases calcium permeability thus, provokes excitotoxicity. Another RNAseq study shows that in repeat containing iPS cells, GluR1, an AMPA-subtype of glutamate receptor, level was twice the level found in control lines (Selvaraj *et al.*, 2018). This suggests that this increase would lead to a calcium entry and a lack of regulation of the calcium permeability by AMPA receptor leading to an AMPA induced excitotoxicity. A LOF study also showed that C9orf72 is involved in vesicle trafficking leading to lysosomal dysfunction. This latter dysfunction would lead to a defect in glutamate receptor turnover at the synaptic cleft resulting in an accumulation of these receptors in the post-synaptic density (Shi *et al.*, 2018).

Table 1-1-3: Genes of ALS

Gene	Protein encoded	Transmission	Regulation of RNA	TDP-43 inclusions	Year of publication
SOD1	Superoxide dismutase 1	AD/AR	Yes	No	1993
TARDBP	Tar-DNA-binding protein-43	AD	Yes	Yes	2008
FUS	Fused in sarcoma	AD/AR	Yes	No	2009
C9orf72	C9orf72	AD	Yes	Yes	2011
ATXN2	Ataxin-2	AD	Yes	Yes	2010
TAF15	TATA-Box Binding Protein Associated Factor 15		Yes	-	2011
UBQLN2	Ubiquilin 2	XLD	No	Yes	2011
OPTN	Optineurin	AD/AR	No	Yes	2010
KIF5A	Kinesin Family Member 5A		No	-	2018
hnRNPA1	Heterogeneous Nuclear Ribonucleoprotein A1	AD	Yes	Yes	2012
hnRNPA2 B1	Heterogeneous Nuclear Ribonucleoprotein A2/B1	AD	Yes	Yes	2013
MATR3	Matrin 3	AD	Yes	Yes	2014
CHCHD10	Coiled-Coil-Helix-Coiled-Coil-Helix Domain Containing 10	AD	No	Yes	2014
EWSR1	EWS RNA Binding Protein 1	AD	Yes	-	2012
TIA1	TIA1 Cytotoxic Granule Associated RNA Binding Protein		Yes	-	2017
SETX	Senataxin	AD	Yes	Yes	2004
ANG	Angiogenin	AD	Yes	Yes	2006
CCNF	Cyclin F		No	Yes	2016
NEK1	NIMA Related Kinase 1		No		2016
TBK1	TANK Binding Kinase 1	AD	No	Yes	2016
VCP	Valosin Containing Protein	AD	No	Yes	2011

SQSTM1	Sequestosome 1	AD	No	Yes	2009
PFN1	Profilin 1	AD	No		2012
TUBB4A	Tubulin Beta 4A Class IVa	AD	No	-	2014
CHMP2B	Charged Multivesicular Body Protein 2B	AD	No	-	2006
SPG11	Spatacsin Vesicle Trafficking Associated	AR	No		2007
ALS2	Alsin Rho Guanine Nucleotide Exchange Factor	AR	No	-	2001
VAPB	Vesicle associated membrane protein (VAMP)/ synaptobrevin associated membrane protein B	AD	No	-	2004
DCTN1	Dynactin 1	AD	No	Yes	2004
SIGMAR1	Sigma non opioid intracellular receptor 1	AR	No	-	2011
PRPH	Peripherin	AR	-	-	2004

1.3 Cellular defects in ALS

1.3.1 RNA dysregulation

Mutations in over more than 40 genes contribute to the etiology of ALS (Chia *et al.*, 2018) (Table 2). Amongst these genes, the major established causal ALS genes are *SOD1*, *TARDBP*, *FUS* and hexanucleotide expansion repeat in *C9ORF72* (Figure 1-4). These genetic discoveries have led to the development of animal models that permitted the identification of key pathobiological insights (Van Damme *et al.*, 2017) and RNA dysregulation appeared to be a major contributor to ALS pathogenesis. Indeed, TDP-43 and FUS are deeply involved in RNA processing such as transcription, alternative splicing and microRNA (miRNA) biogenesis (Buratti *et al.*, 2004; Buratti *et al.*, 2010; Polymenidou *et al.*, 2012). Mutations in *C9ORF72*, lead to a toxic mRNA GOF through RNA foci formation and altered activity of RNA-binding proteins (Barker *et al.*, 2017). In addition to these four major ALS genes, other ALS genes including ataxin-2 (*ATXN2*) (Ostrowski *et al.*, 2017), TATA-box binding protein associated factor 15 (*TAF15*) (Ibrahim *et al.*, 2013),

heterogeneous nuclear ribonucleoprotein A1 (*hnRNPA1*) (Dreyfuss *et al.*, 1993) heterogeneous nuclear ribonucleoprotein A2 B1 (*hnRNPA2 B1*) (Alarcon *et al.*, 2015), matrin 3 (*MATR3*) (Coelho *et al.*, 2015), Ewing's sarcoma breakpoint region 1 (*EWSR1*) (Duggimpudi *et al.*, 2015), T-cell-restricted intracellular antigen-1 (*TIA1*) (Forch *et al.*, 2000), senataxin (*SETX*) and angiogenin (*ANG*) (Yamasaki *et al.*, 2009), play critical role in RNA processing.

For example, SOD1 can interact with the 3'-Untranslated region (UTR) of several mRNA and affect their function and stability like vascular endothelial growth factor (VEGF) or Neurofilament light chain (NFL) (Chen *et al.*, 2014; Lu *et al.*, 2007; Menzies *et al.*, 2002). Indeed, one study shows that mutant SOD1 can bind the *NFL* mRNA leading to neurofilament aggregation and neurite degeneration in iPS cells (Chen *et al.*, 2014; Menzies *et al.*, 2002). Actually, SOD1 has been reported to interact with TDP-43 to regulate NFL mRNA stability, indeed, TDP-43 has been found to stabilize NFL mRNA by binding its 3' UTR (Strong *et al.*, 2007; Volkening *et al.*, 2009). This suggests a common role in mRNA stability regulation between TDP-43 and SOD1 although, Rotem and colleagues compared transcriptome of both TDP-43 and SOD1 mouse primary spinal cord motoneurons and found that most genes misregulated in a model was not in the other, only a few genes were affected in both models (Rotem *et al.*, 2017).

About TDP-43, it has been reported to regulate other known ALS genes such as *FUS* and ataxin 2 (*ATXN2*) (Polymenidou *et al.*, 2011; Sephton *et al.*, 2011; Tollervey *et al.*, 2011). An RNAseq study revealed that TDP-43 is involved in the regulation of 239 RNAs and many of these are involved in the synaptic formation and function (Polymenidou *et al.*, 2011). Indeed, neurexin, neuroligin, GABA and AMPA receptors subunits are amongst these genes (Narayanan *et al.*, 2013; Polymenidou *et al.*, 2011; Sephton *et al.*, 2011). This can explain why TDP-43 models show synaptic defects (Armstrong *et al.*, 2013a; Feiguin *et al.*, 2009; Handley *et al.*, 2017). TDP-43 also acts as a splicing regulator and has been found to regulate stathmin 2 (*STMN2*) (Klim *et al.*, 2019). It has been reported that a loss of TDP-43 leads to the exon 2a transcription encoding a stop codon and a polyadenylation signal producing a truncated protein. Reduced levels of *STMN2* have been found in both sporadic and familial cases of ALS. In a similar way, it a recently been discovered that *UNC13a* is also regulated by TDP-43 and is expressed in the nervous system, more precisely, at the synaptic level in the central nervous system and at the neuromuscular junction. It is involved in vesicle priming steps prior to synaptic vesicle fusion (Augustin *et al.*, 1999; Bohme *et al.*, 2016; Deng *et al.*, 2011b; Lipstein *et al.*, 2017). A loss of TDP-43 regulation leads to the transcription of a cryptic exon leading to decreased levels of *UNC13a* (Brown *et al.*, 2022; Ma *et al.*, 2022).

FUS is also an RNA regulator it has been found to regulate neuronal maintenance and survival related genes splicing (Lagier-Tourenne *et al.*, 2012). A LOF of *FUS* leads to 300 genes dysregulation in mice brain. In the same way, many genes of the previous study were found downregulated in FUS depleted neuronal cells (Reber *et al.*, 2016). In these cells, FUS depletion affected minor intron containing genes including genes involved in neurogenesis (*PPP2R2C*), dendritic development (*ACTL6B*) and action potential transmission in skeletal muscle (*SCN8A* and *SCN4A*) (Reber *et al.*, 2016).

C9orf72 also has a cytotoxic role when mutated through the RNA foci accumulating in the cell nucleus (DeJesus-Hernandez *et al.*, 2011; Wojciechowska *et al.*, 2011). The foci can bind RNA binding proteins (RBP) like hnRNP-A3, TDP-43 or FUS, engendering even more RNA dysregulation (Donnelly *et al.*, 2013; Lee *et al.*, 2013; Mori *et al.*, 2013b). Antisense oligonucleotides have been decreasing the amount of RNA foci formation leading to a reversed regulation of the altered RNAs (Donnelly *et al.*, 2013; Lagier-Tourenne *et al.*, 2013). Dipeptide repeat proteins (DPR) produced in *C9orf72* pathology have also been involved in RNA dysregulation by creating aggregated sequestrating cytoplasmic proteins (Freibaum *et al.*, 2017). Poly-GR have been found to colocalize with ribosomal proteins and transcription factor suggesting a ribosomal dysfunction and a defective translation (Zhang *et al.*, 2018d). C9orf72 effects on cellular processes will be described further in section 1.6.

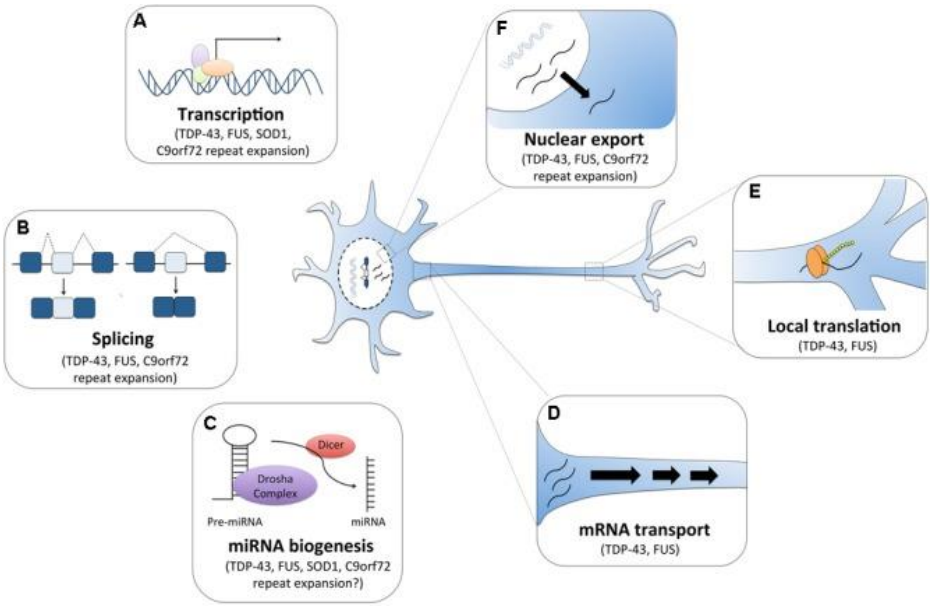

Figure 1-4: Defective RNA processes in ALS.

(A) mutations in ALS genes *SOD1, TDP-43, FUS and C9orf72* can alter gene expression. (B) The RNA binding proteins TDP-43 and FUS can affect global splicing machinery. Dipeptide repeat proteins from C9orf72 intronic expansion can also alter splicing patterns of specific RNAs. (C) TDP-43, FUS, and dipeptide proteins can also promote microRNA biogenesis as components of the Drosha and Dicer complexes. TDP-43 and FUS also alter mRNA transport (D) and local translation (E). (F) TDP-43 and FUS predominantly reside in the nucleus, but when mutated they are can mislocalization to the cytoplasm where they bind and regulate different sets of RNAs including the export and mislocalization of other transcripts to the cytoplasm. Poly-PR dipeptide can also bind nuclear pores channels blocking the import and export of molecules. Adapted from (Butti *et al.*, 2018). (Authorized by CC BY 4.0 licence, Creative commons).

1.3.2 Protein dysregulation

Misfolded proteins are constantly produced in physiological conditions, that is why cells have several checkpoint and degradation mechanisms to prevent toxicity arising upon these defective proteins. The persistence of these problematic proteins reflects a defectiveness is these clearing mechanisms. The rupture of cellular homeostasis is the result of the imbalance between protein synthesis and clearance. Protein clearance can be achieved by two separate pathways: via the proteasome and via lysosomes. The proteasome handles the degradation of proteins tagged with ubiquitin (Tanaka, 2013), while autophagy implicates lysosomes and the enzymes contained to mediate protein degradation. Autophagy process and defects in ALS will be described further in section 1.3.5.

Protein inclusions are a key feature of ALS and historically, protein aggregates have been found in motoneurons, spinal cord, cerebellum, frontal and temporal lobes in the cortex (Blokhuis *et al.*, 2013; Gotzl *et al.*, 2016; Kabashi *et al.*, 2006; Medinas *et al.*, 2017; Ruegsegger *et al.*, 2016). ALS protein inclusions are heterogenous, TDP-43 positive are the most common inclusions since more than 99 percent of all ALS cases present TDP-43 containing aggregates, only *SOD1* and *FUS* mutants seem to avoid this type of aggregates but have been characterized by SOD1 and FUS positive aggregates (Kwiatkowski *et al.*, 2009; Mackenzie *et al.*, 2007; Vance *et al.*, 2009). p62/sequestosome 1 is also frequently found in protein aggregates in ALS patients (Al-Sarraj *et al.*, 2011; Mizuno *et al.*, 2006). Ubiquitin positive inclusions have also been found in motoneurons and cortical brain region of ALS patients (Lowe, 1994; Walling, 1999). These observations correlate with the cognitive decline in patients. In ALS, TDP-43 is reported as truncated in the N-terminal part of the protein while its C-terminal is found hyperphosphorylated and ubiquitylated in brain tissues, although, these modifications are not found in in spinal cord tissue (Feneberg *et al.*, 2021).

Another problematic category of protein found dysregulated in ALS are the RNA binding proteins (RBP). A dysregulation of these proteins can lead to defective post-translational modifications, nucleocytoplasmic traffic, as well as aggregation and sequestration of RNAs (Xue *et al.*, 2020). Indeed, several ALS genes are encoding RBP, such as TDP-43, FUS, hnRNP A1, hnRNP A2/B1, TAF15, TIA1, EWSR1, ATXN2 (Al-Chalabi *et al.*, 2017). Mutant RBPs have a higher tendency to aggregate or mislocalize. Mutations in these genes lead to their encoded protein dysregulation and engender RNA dysregulation that is describe in the previous section.

1.3.3 Axonal transport defects

Neuron polarization makes axonal transport a crucial mechanism to maintain cellular homeostasis and neuronal survival. Anterograde and retrograde transport of cargo containing RNAs, proteins or organelles for example require a functioning microtubule network. Anterograde transport is mediated by kinesin motor protein and retrograde transport is mediated by dynein-1 protein. Genes involved in both transport direction have been reported to be mutant in some ALS cases: KIF5A and DCTN1 are a kinesin family member and a regulator of the dynein complex respectively which indicate a strong implication of this pathological mechanism in the disease (Brenner *et al.*, 2018; Munch *et al.*, 2007; Nicolas *et al.*, 2018; Zhang *et al.*, 2019a). Furthermore, due to its RNA dysregulation effect, FUS can disrupt kinesin family mRNA processes. Indeed, *FUS* mutations have been linked to the dysregulation of *KIF5C, KIF1B* and *KIB3A* mRNAs (Hoell

et al., 2011). Mice mutant for *SOD1* have showed a defective dynein dependant pathway, linking *SOD1* mutations to retrograde transport defects (Bilsland *et al.*, 2010; Kieran *et al.*, 2005).

Besides motor proteins, axonal transport requires a functioning microtubule and neurofilament network. Two studies have linked the accumulation of neurofilaments in neurons to both fALS and sALS (Munoz *et al.*, 1988; Williamson *et al.*, 1998). One of them showed that neurofilament deletion in a *SOD1* mouse model leads to a neuroprotective effect (Williamson *et al.*, 1998). Kinesin and dynein defects can both lead to a neurofilament accumulation or disruption since both proteins are involved in the neurofilaments oligomers transport (Prahlad *et al.*, 2000; Sunil *et al.*, 2012). Phosphorylation of neurofilament heavy chain is a hallmark of ALS, has become a biomarker of C9orf72 pathology, and can provoke a disruption of axonal transport (Gendron *et al.*, 2017; Mizusawa *et al.*, 1989; Munoz *et al.*, 1988). Similarly, peripherin (PRPH), which also has a role in neurofilament regulation has been found mutant in ALS cases showing again a link between axonal transport and ALS (Corrado *et al.*, 2011; Gros-Louis *et al.*, 2004; Leung *et al.*, 2004). Moreover, mutations in the *TUBA4A* gene have been associated with ALS in 2014 (Smith *et al.*, 2014). TUBA4A is involved in microtubule dynamic and stability and mutations in the gene have been found to have a dominant negative effect. Although it is expressed in the nervous system, its role in motoneurons is still unknown (Smith *et al.*, 2014).

As described, axonal transport is linked to ALS either directly with genes mutations involved in the disease, such as TUBA4A, KIF5A or DCTN1, either through gene dysregulation leading to a disruption of the axonal traffic. Recently, TDP-43 has been linked to axonal transport disruption by creating RNP condensates containing RNAs and G3BP1 leading to interference in axonal and pre-synaptic proteins synthesis (Altman *et al.*, 2021). Indeed, TDP-43 can be found in the axons since it is involved in mRNA transport to the synaptic end (Alami *et al.*, 2014; Fallini *et al.*, 2012).

1.3.4 Nucleocytoplasmic transport defects

Small proteins (40kDa or less) can freely diffuse across the nuclear membrane. Although larger proteins require an active transport between the cytoplasm and the nucleus. Nucleocytoplasmic transport (NCT) refers to this active mode of transport. NCT requires three major components: a nuclear pore complex (NPC), nuclear transport receptors and a RAN GTP gradient to be efficient. Proteins using this transport need to present either a nuclear localization sequence (NLS) or a nuclear export signal (NES) or both to be carried through the NPC.

Several proteins involved in the NCT, including importins, RAN Gap and nucleoporins, have been found mislocalized and aggregated in post-mortem brain and spinal cord tissues from ALS

patients (Aizawa *et al.*, 2019; Chou *et al.*, 2018; Coyne *et al.*, 2020; Kinoshita *et al.*, 2009; Saberi *et al.*, 2018; Shang *et al.*, 2017; Solomon *et al.*, 2018; Zhang *et al.*, 2015).

Cellular abnormalities of the NCT have also been reported in different models of ALS both *in vitro* and *in vivo* (Chou *et al.*, 2018; Jovicic *et al.*, 2015; Kinoshita *et al.*, 2009; Lee *et al.*, 2016; Shang *et al.*, 2017; Solomon *et al.*, 2018; Woerner *et al.*, 2016). Indeed, nucleoporin nup107 showed abnormal staining pattern in a $(GGGGCC)_{58}$ expressing drosophila and in spinal cord of a *SOD1* mouse model (Freibaum *et al.*, 2015; Shang *et al.*, 2017). Expression of this nup107 along with 7 other nucleoporins were found to be decrease in C9orf72 iPS cells (Coyne *et al.*, 2020). More recently, CHMP7 expression, which is involved in NPC quality control was found increased in both C9orf72 iPS cells, in sporadic iPS cells as well as in post-mortem patient motor cortex (Coyne *et al.*, 2021). In addition, it was reported that inhibiting the nuclear export of CHMP7 would lead to a decreased TDP-43 pathology in neurons. Lastly, knocking down CHMP7 led to a better RAN GTPases localization, improvements in TDP-43 regulated mRNAs expression and a decreased glutamate induced neuronal death (Coyne *et al.*, 2021). Although these studies do not all converge to the same conclusions and their findings can be contradictory. Some study reports no abnormality about the RAN Gap protein localization in patient motor cortex tissue while others find an abnormal nuclear localization or even cytoplasmic upregulation of the protein (Saberi *et al.*, 2018; Shang *et al.*, 2017; Zhang *et al.*, 2015). Again, importin-β1 has been reported without any abnormality in C9orf72 mutant tissue while a cytoplasmic localization or a nuclear depletion are observed in sALS patient spinal cord tissue (Aizawa *et al.*, 2019; Kinoshita *et al.*, 2009; Saberi *et al.*, 2018). It has been showed recently that arginine rich dipeptides can bind importin-β which can disrupt the NCT in different models (Hayes *et al.*, 2020; Hutten *et al.*, 2020).

Together, these data make it difficult to know if the NCT defects observed in post-mortem tissues are a cause or a consequence of the disease.

1.3.5 Autophagy

As seen previously, the presence of protein aggregates and their mislocalization suggest a major defect in protein homeostasis. Autophagy is a key pathway for protein and organelle degradation involving lysosomes using three different pathways: macroautophagy involving membrane enveloping proteins or an organelle called an autophagosome and its fusion to a lysosome to degrade and recycle components. Microautophagy is the process by which proteins are degraded by engulfment by lysosomes (Li *et al.*, 2012). The third autophagic process is the chaperone mediated autophagy which involved chaperone proteins to bring protein to the lysosome (Kaushik

et al., 2012). This is a selective degradation pathway depending on the presence of an amino acid motif contained in the protein sequence that is recognized by chaperone proteins to trigger the degradation (Cuervo *et al.*, 2014).

Dysregulation of autophagy has been reported in both fALS and sALS, making it a key feature of the cellular defects of ALS. A study reports that sALS patients present protein inclusions positive for LC3 and SQSTM1 in their motoneurons (Sasaki, 2011). SQSTM1 inclusions were also positive for Ubiquitin which reveals a dysregulation of the protein clearance pathways (Mizuno *et al.*, 2006). Also, mitochondrial abnormalities such as enlarged mitochondria or accumulation of dysmorphic form of the organelle have been reported in both spinal cord cells, intramuscular nerves and skeletal muscles of sALS patients (Atsumi, 1981; Chung *et al.*, 2002; Napoli *et al.*, 2011; Sasaki *et al.*, 1996; Wiedemann *et al.*, 1998).

In addition to these findings, studies have been linking mutations in genes of autophagy to ALS disease. Indeed, Sequestosome-1 (SQSTM1) has been found has a major element of neuronal and glial cytoplasmic inclusions in ALS patients (Kuusisto *et al.*, 2001; Zatloukal *et al.*, 2002). This gene is involved in 1 to 3.5 percent of all ALS cases (Fecto *et al.*, 2011; Le Ber *et al.*, 2013; Teyssou *et al.*, 2013). SQSTM1 protein has several domains but mainly, the two domains harbouring ALS mutations are the ubiquitin associated domain (UBA), involved in ubiquitin recognition and LC3 interacting region (LIR) which enables the interaction with the ATG8 family of protein located on the autophagosome (Lippai *et al.*, 2014; Pankiv *et al.*, 2007). These mutations prevent from ubiquitin recognition when in the UBA domain and LC3 recognition important for substrate delivery to autophagosome when in the LIR domain, impeding SQSTM1 activation leading to an altered autophagy pathway (Fecto *et al.*, 2011; Ichimura *et al.*, 2008; Le Ber *et al.*, 2013; Pankiv *et al.*, 2007; Rea *et al.*, 2013; Teyssou *et al.*, 2013). Mutations in the promoter regions have also been reported in ALS patients leading to a LOF of the gene showing how this gene and autophagy have a role in ALS pathology (Rubino *et al.*, 2012).

Furthermore, C9ORF72 patients show SQSTM1 positive inclusions associated with ubiquitin in neurons, glial cells and muscle fibers (Al-Sarraj *et al.*, 2011; Mackenzie *et al.*, 2014; Troakes *et al.*, 2012; Turk *et al.*, 2014). C9orf72 has been linked to autophagy but its role will be described further in section 1.6.4.

Another autophagy related gene found mutant in around 1 percent of ALS cases in TANK binding kinase 1 (TBK1) (Cirulli *et al.*, 2015; Freischmidt *et al.*, 2015). Patients tissue analysis show reduced expression of TBK1 mRNA suggesting a LOF mechanism. TBK1 is in charge of the phosphorylation of autophagy receptors like optineurin (OPTN) or SQSTM1 for example which

confers it a role in autophagy regulator (Heo *et al.*, 2015; Matsumoto *et al.*, 2015; Richter *et al.*, 2016; Wild *et al.*, 2011). TBK1 is involved on the early stages of autophagy by phosphorylating SMCR8 which makes a complex with C9orf72 and WDR41, this complex being involved in initiation of the autophagy process (Sellier *et al.*, 2016).

Other genes found mutant in ALS patients have been reported to have a link with all stages of autophagy, C9orf72 affecting the early stage as seen previously, SQSTM1 and CHMP2 affecting intermediate stages and VCP affecting late stage. Indeed, charged multivesicular body protein 2 (CHMP2) is involved in membrane deformation necessary for the autophagosome formation and endosomal trafficking. Valosin containing protein (VCP) has a role in autophagosome maturation and mutations in this gene lead to a disruption of the maturation and a hindering of the fusion between the autophagosome and the lysosome (Ju *et al.*, 2009; Tresse *et al.*, 2010). Ubiquilin 2 (UBQLN2) is also found mutant in some ALS cases, it acts as an autophagy receptor by binding LC3 on autophagosome (Daoud *et al.*, 2012; Deng *et al.*, 2011a; Millecamps *et al.*, 2012; N'Diaye *et al.*, 2009; Rothenberg *et al.*, 2010). Lastly, Optineurin (OPTN) found mutant in around 3 percent of fALS and 1 percent of sALS can also act as an autophagy receptor by binding ubiquitinated protein and LC3 domain (Korac *et al.*, 2013; Richter *et al.*, 2016; Wong *et al.*, 2014). OPTN can interact with SQSMT1 as well to accelerate the autophagic flux (Liu *et al.*, 2014b). Mutations in this gene mostly affect the ubiquitin binding domain reducing the autophagic flux (Evans *et al.*, 2020).

1.3.6 Oxidative stress

Oxidative stress results from the imbalance between the production of Reactive oxygen species (ROS) and their clearance. The discovery of the causative link between *SOD1* mutations and ALS suggested a strong implication of oxidative stress in ALS pathogenesis (Rosen *et al.*, 1993). Post-mortem brain from patients showed proof of oxidative damages (Ferrante *et al.*, 1997). This observation led to the development of potential biomarkers that have been found to have increased level in several patient fluids. For instance, 8-hydroxy-2'-desoxyguianosine, a nucleic marker of oxidative stress damages, has been found with higher levels in cerebrospinal fluid, plasma and urine of both fALS and sALS patients, while glutathione level, an antioxidant is decreased in patient's motor cortex (Bogdanov *et al.*, 2000; Ferrante *et al.*, 1997; Konovalova *et al.*, 2019). In addition, other oxidative stress products such as Malone dialdehyde and 4-hydroxynonenal were found increased in patient's plasma (Baillet *et al.*, 2010; Blasco *et al.*, 2017; Moumen *et al.*, 1997).

Aging brain becomes more sensitive to oxidative stress and antioxidant functions decreased (Andersen, 2004; Tarafdar *et al.*, 2018). Indeed, oxidative stress can damage neurons and in ALS, motoneurons through various pathways. It can generate post-translational modifications such as amino acid alteration, for example, creating a cysteine bond through oxidation engendering a protein misfolding and triggering its aggregation (Oka *et al.*, 2013; Serebryany *et al.*, 2016). TDP-43 for example can undergo a cysteine disulphide crosslink reaction which promotes its cytoplasmic aggregation and decrease its solubility (Cohen *et al.*, 2012). Protein aggregation can also be triggered by alteration of chaperone protein activity, by preventing the interaction between chaperones and heat shock proteins disrupting protein translation (Karri *et al.*, 2019). Nucleo-cytoplasmic transport can be disturbed as well by oxidative stress: Nup153 and importin-β have been found to be relocated upon oxidative stress (Kodiha *et al.*, 2004). Besides, high levels of oxidative DNA and DNA repairment damages lead to p53 activation resulting in apoptosis in *SOD1* and *FUS* neuron cell lines and in C9-patient derived iPS cells (Barbosa *et al.*, 2010; Lopez-Gonzalez *et al.*, 2016; Wang *et al.*, 2018). A non-cell-autonomous effect has also been reported with derived astrocytes carrying C9orf72 mutation triggering oxidative stress in co-cultured motoneurons (Birger *et al.*, 2019).

Nuclear factor erythroid-2-related factor 2 (Nrf2) have been reported as neuroprotective in oxidative stress condition and have been found to have low level of expression in motoneurons from SOD1 patients and in primary motor cortex and spinal cord from post-mortem patient tissues (Sarlette *et al.*, 2008; Wu *et al.*, 2006). A *SOD1* mutant mouse model crossed with an astrocyte specific overexpressing Nrf2 mouse showed a delayed disease onset and an extended survival rate underlining Nrf2's neuroprotective role (Vargas *et al.*, 2008). Although, a similar study only found modest impact of Nrf2 overexpression in a mouse model as well (Guo *et al.*, 2013). TARDBP mutations have also been linked to a decrease of Nrf2 expression and activation, leading to an enhancement of oxidative stress (Duan *et al.*, 2010; Tian *et al.*, 2017). This pathway is still investigated as a potential therapeutic avenue for ALS (Arslanbaeva *et al.*, 2022).

Lastly, mutations in *TARDBP* and *C9orf72* have been reported to have on mitochondria activity and bioenergetics to promote cell death (Onesto *et al.*, 2016). More recently, *C9ORF72* patients fibroblasts showed a 2 to 3 folds increase of lipid and protein oxidation and mitochondrial dysfunctions (Alvarez-Mora *et al.*, 2022).

1.4 Synaptic defects in ALS

1.4.1 Synaptic transmission in normal conditions

Neuron's primary role is the message transmission at the synapse using electrical potentials created by ion gradients. There are two types of synapses: electrical and chemical. Electrical synapses can be found in the whole nervous system but are in minority compared to the chemical ones. They are composed of gap junctions allowing the influx of ATP or metabolite directly from the presynaptic neuron to the postsynaptic one. The message transmission is particularly fast, so the main role of these synapses is to synchronize the electrical activity of a population of neurons like the neurons in the brainstem that control our breathing system. Chemical synapses present a bigger space between the two communicating neuron, this space is the synaptic cleft. Message transmission involves synaptic vesicles in the presynaptic neuron containing the neurotransmitter that gets released in the synaptic cleft by membrane fusion.

Neuronal transmission follows a sequence and is triggered by an action potential in the presynaptic end, which leads to the opening of calcium canals letting the calcium in. This calcium allows the fusion of the membranes between the synaptic vesicles and the presynaptic membrane, this releases the neurotransmitter in the cleft by exocytosis. The neurotransmitter is getting bound on its receptor on the post synaptic neuron that provokes a current. The neurotransmitter can be either recaptured by the presynaptic neuron or a glial cell around either getting enzymatically degraded at the synaptic cleft and the synaptic vesicles get recycled in the presynaptic neuron. Synaptic homeostasis is highly regulated and involves several cell types like glial cells that have a role in neurotransmitter recapture hence its concentration, elimination of impaired synapses and maintaining the myelin sheath quality (Barres *et al.*, 2000; von Bernhardi *et al.*, 2016).

Neuronal transmission using chemical synapses involves neurotransmitters that can be excitatory or inhibitory. Amongst the different types of neurotransmitters, GABA and glycine are the most common inhibitory ones while acetylcholine and glutamate are the most common excitatory ones. Neurotransmitters can use two types of receptors: ionotropic and metabotropic. Ionotropic are ionic canals that can bind the neurotransmitter and open to let the ion current while metabotropic receptors bind the neurotransmitter but must activate a G protein that triggers the opening of an ionic canal via a signaling pathway.

A subtype of these chemical synapses are the neuromuscular junctions (NMJ). NMJ is the synapse between the motoneuron and the muscle. The neurotransmitter involved is the

acetylcholine and it is degraded by the acetylcholinesterase enzyme. At this junction, some low amplitude currents are observed: miniatures end plate currents (mEPCs). These currents are spontaneous signals sent by the motoneuron to the muscle, it is less than 1mV so it does not trigger any action potential. Their role is to keep the NMJ active and healthy. The NMJ is a tripartite system involving a pre-synaptic part, the motoneuron, a post-synaptic part on the muscle and glial cells. Schwann cells are the glial cells at the NMJ and is involved in the support of its function and structure (Ko *et al.*, 2015). The pre-synaptic part of the NMJ is enriched in mitochondria and has a developed endosomal network to ensure the formation, accumulation and recycling of the synaptic vesicles. These synaptic vesicles are concentrated in or close to active zones that are dense in electron microscopy imaging. Active zones are where exocytosis of the acetylcholine neurotransmitter happens and contain voltage gated calcium channels (VGCC) involved in the calcium influx necessary for the docking of the synaptic vesicle and release of acetylcholine (Slater, 2017; Wood *et al.*, 2001). Docking, fusion and neurotransmitter release happen with the involvement of soluble N-ethylmaleimide-sensitive factor attachment receptors (SNAREs). Besides, the post-synaptic part presents membrane folding called synaptic crests. Top of these crests contains clusters of nicotinic acetylcholine receptors and the bottom of the crests presents voltage dependent sodium channels that open after depolarization engendered by the binding of acetylcholine on its receptor (Yang *et al.*, 1991). Organization of the acetylcholine receptors in this post synaptic part is regulated by a pathway involving agrin secreted by the motoneuron, agrin bind to its receptor low-density lipoprotein receptor related protein 4 (LRP4) that induces the phosphorylation of its coreceptor muscle specific kinase (MuSK) to create clusters of acetylcholine receptors (Sanes *et al.*, 2001) (Darabid *et al.*, 2014).

1.4.2 Synaptic transmission in ALS patients

Synaptic activity in ALS is largely studied since this process is defective in the disease. Indeed, patients show a decrease of amplitude of miniature endplate potentials and currents (Maselli *et al.*, 1993). Additionally, axonal integrity was reported to be affected in ALS leading to a dysfunctional NMJ (Kennel *et al.*, 1996). A study using patient samples have demonstrated that NMJs from ALS patients show major alterations in morphology in the three components: axon terminal, post synaptic structure and terminal Schwann cell (Bruneteau *et al.*, 2015). These alterations have been observed in both patients in early stage of the disease and late stage, suggesting that the NMJ defects appear early in the process. Denervation had already been reported in previous studies (Bjornskov *et al.*, 1975; Tsujihata *et al.*, 1984; Yoshihara *et al.*, 1998). Recently, a study has compared NMJ from ALS patients and from control individuals (Ding *et al.*,

2022). They showed that 67 percent of ALS patient NMJs showed evidence of partial denervation or reinnervation and 18 percent evidence of denervation compared to controls who had 92 percent of normal NMJs. In addition, patient nerve terminal boutons were reduced in average size. Muscle fibers from patients were less responsive to human motoneuron innervation since they produced less acetylcholine receptors. Further analysis of these samples revealed a delocalization of MuSK which is a part of the n-agrin receptor and thus a failure to respond to n-agrin signalling. Altogether, results from this study suggest that the massive NMJ denervation observed in ALS patients is a consequence of a defect in the agrin-LRP4-MuSK pathway.

1.4.3 Excitotoxicity

Excitotoxicity is the toxicity induced by glutamate in neurons in case of prolonged exposure to it. Glutamate is mostly recaptured by the excitatory amino acid transporter 2 (EEAT2). Excitotoxicity can be due to a lack of recapture or an increase of the release of glutamate. Accumulating glutamate in the synaptic cleft provokes an excessive activation of the receptors leading to a calcium overload and a toxic hyperactivation of the neurons (Cleveland *et al.*, 2001; Pasinelli *et al.*, 2006). AMPA receptors (AMPAR) have also been identified as a cause of excitotoxicity. AMPA receptors are ionotropic and open when bounded with glutamate. AMPAR have four different subunits, GluA1-4, each subunit conferring different biophysical and molecular properties (Gan *et al.*, 2015).

More precisely, GluA2 has the ability to make GluA2 containing AMPAR calcium impermeable (Isaac *et al.*, 2007). An Adenosine DeAminase acting on RNA 2 (ADAR2) is in charge of replacing an adenosine into an inosine in the GluA2 mRNA sequence changing a Q (glutamine) codon to a R (arginine) codon in the GluA2 protein sequence (Hideyama *et al.*, 2012). This edition makes the GluA2 subunit impermeable to calcium. ALS patients show a lack of ADAR2 leading to an increase of unedited GluA2 protein sequence expression which involves a higher permeability to calcium in neurons having GluA2 subunit containing AMPAR (Greger *et al.*, 2002). A higher calcium permeability leads to excitotoxicity and neuronal death. This edition defect on GluA2 sequence has been reported in spinal motoneurons from ALS patients (Kawahara *et al.*, 2003a).

Patch clamp technique in *SOD1* model have shown that mutant motoneurons were firing more than control and that it would be explained by a decrease of the delayed-rectifier potassium current amplitudes when compared to controls. C9orf72 and TDP-43 motoneurons have shown more firing as well but explained by a decrease of potassium and sodium current amplitudes.

Other studies have reported a decreased sensitivity of *C9orf72, SOD1 and TDP-43* mutant motoneurons at late timepoints. This could be explained by a progressive theory saying that mutant motoneurons go from an early hyperexcitability that changes into a nearly inexcitability (Devlin *et al.*, 2015). Devlin and colleagues also stated that neurons derived from C9orf72 patients iPS cells would show an early hyperxcitability indicating that this process is an actor of the initiation of the disease leading to a later loss of the motoneurons. This data has been confirmed recently with a similar conclusion about an early hyperexcitability in C9orf72 patients derived motoneurons (Burley *et al.*, 2022).

1.5 Model organisms to study ALS

Model organisms have a key role in disease study and drug discovery although no model can replicate all human physiology. That is why using every different model can give interesting insights into the disease mechanisms and put together, these data can give rise to new discoveries. Models for *C9orf72* will be further described in section 1.7.

1.5.1 iPS cells

Human induced pluripotent stem (hiPS) cells have brought new opportunity to model ALS in disease relevant cell types and in non-accessible cells like neurons and glial cells. It has been used to model cortical and spinal motoneurons in ALS research. This model preserves the genetic background from patients and allows to study the mutations in the clinically relevant conditions.

iPS cells have been used to model fALS, *C9orf72* and *TDP-43* mutations are the most studied. Indeed, *SOD1* iPS studies have been showing ALS relevant phenotypes such as a decreased survival cell rate and a decrease of both cell soma size and neurite length in derived motoneurons in a *SOD1A4V* line (Kiskinis *et al.*, 2014). Impaired axonal growth and neurofilament dynamics were also reported in *SOD1* lines (Chen *et al.*, 2014; Kim *et al.*, 2020). Although, conclusions drawn using these lines are not similar to the ones obtain using rodent models that display more SOD1 misfolding phenotypes (Joyce *et al.*, 2011). *FUS* iPS cells can reproduce the mislocalization as well as DNA damage repair and splicing alterations, axonal RNA transport defects and NMJ instability (Guo *et al.*, 2017; Higelin *et al.*, 2016; Ichiyanagi *et al.*, 2016; Marrone *et al.*, 2018; Naumann *et al.*, 2018; Picchiarelli *et al.*, 2019). In *TDP-43* carrying mutation lines, higher insoluble TDP-43 levels were found, despite these higher levels, clear aggregates generally could not be reproduced. IPS cells-derived motoneurons showed impaired neurofilament organization and axonal vesicular trafficking that can explain the reduced growth

and survival of these cells (Kreiter *et al.*, 2018; Serio *et al.*, 2013). C9orf72 cell lines can reproduce both toxic GOF, RNA foci formation and DRP aggregation as well as the reduced levels of expression although a major signature of the C9orf72 pathology, TPD-43 mislocalization and aggregation fails to be reproduced (Almeida *et al.*, 2019; Donnelly *et al.*, 2013; Gendron *et al.*, 2017; Sareen *et al.*, 2013). These cell lines have demonstrated electrophysiological defects such as an impaired firing activity, a higher sensitivity to glutamate toxicity, alteration in the nucleo-cytoplasmic transport and defects in both vesicular trafficking (Dafinca *et al.*, 2016; Donnelly *et al.*, 2013; Freibaum *et al.*, 2015; Sareen *et al.*, 2013; Zhang *et al.*, 2015).

iPS cells strength, compared to genetics models, is that it can model sALS. Fujimori and colleagues have been modelling and comparing iPS derived motoneurons from *SOD1, FUS, TDP-43* patients and 32 cell lines from sALS patients and found that these lines could recapitulate the main ALS features including a higher cell death rate, an abnormal neural length and protein aggregation which correlated with the clinical heterogeneity (Fujimori *et al.*, 2018). In 2019, sALS patients derived motoneurons showed an accumulation of glutamate receptors and a defect in autophagosome formation, these conclusions correlate with previous findings from the same team on *C9orf72* patients derived motoneurons that showed an accumulation of glutamate receptors as well with an excitotoxicity and defects in DPR clearance (Shi *et al.*, 2019; Shi *et al.*, 2018).

iPS cells is a useful tool for drug screen and drug development in pre-clinical stage. Indeed, *C9orf72* and *SOD1* cell lines showed an efficiency during ASOs development (Miller *et al.*, 2020; Sareen *et al.*, 2013). Also, ezogabine, an FDA drug approved for epilepsy was found to decrease cortical and spinal motoneurons excitability in ALS which led to a clinical trial (Wainger *et al.*, 2021). Although, this trial did not give significant results. Last, Shi and colleagues could identify 3K3A-APC as a potential treatment giving promising results such as a rescue of the proteostasis and a decrease of glutamate receptors accumulation in both *C9orf72* patients and sALS patients derived motoneurons (Shi *et al.*, 2019).

iPS major downfalls are the variability of purity and maturity of the cells giving confounding results, indeed, *C9orf72* hiPS cells derived motoneurons exhibited hyperexcitability in one study while others reported a hypoexcitability in the same model (Dafinca *et al.*, 2020; Devlin *et al.*, 2015; Wainger *et al.*, 2014). These data were in opposition but a longitudinal study reported that the motoneurons initially show a hyperexcitability turns into a hypoexcitability phenotype upon an extended culture period (Dafinca *et al.*, 2020).

1.5.2 Mouse

Mouse is the first choice to model human diseases. It is the reference model for pre-clinical studies. Mouse being mammals, they show a great genetics homology with the human genome as well as a great conservation of the biological processes. Its relatively long lifespan allows modeling neurodegenerative diseases that appear with time. The mouse genome is fully known and genetics tools have been set up to edit it.

Since the discovery of *SOD1*, mouse models have been generated and have brought great new insights into affected cellular and molecular processes. Indeed, *SOD1*-G93A was the first model to study ALS (Gurney *et al.*, 1994) and it is still one of the most used models in laboratories. Since then, several mutations have been modelized in mouse like G37R mutation (Wong *et al.*, 1995) or G85R mutation (Bruijn *et al.*, 1997). These mice model resume ALS main features such as progressive paralysis phenotype, axonal denervation, motoneuron death and protein aggregation (Philips *et al.*, 2015). In addition, these models have brought new key insights about protein defects like aggregation and misfolding, excitotoxicity, axonal transport defects (Ilieva *et al.*, 2009). *SOD1* mice have also been a key model in the pre-clinical trials for Riluzole and Edaravone (Gurney *et al.*, 1998; Ito *et al.*, 2008; Traynor *et al.*, 2006; Venkova-Hristova *et al.*, 2012; Zoccolella *et al.*, 2009).

Mouse model for TDP-43 have been showing ALS features such as motor and cognitive defects and motoneuron loss although they did not show any paralysis or premature death (Arnold *et al.*, 2013; Swarup *et al.*, 2011). A hindrance to studying TDP-43 pathology in mice comes from the fact that the RNAs bound and processed by TDP-43 in Human are not the same as in mice, plus, their processing differs between species.

FUS mouse models have also been generated to investigate the part of the loss of function in the disease due to the loss of nuclear localization of FUS and the part of the toxic cytoplasmic localization in the pathological mechanism. Mouse model with *FUS* knockout show a postnatally lethal phenotype because of respiratory failure in the newborn pups and motoneuron loss (Scekic-Zahirovic *et al.*, 2016). Heterozygous mice showed FUS cytoplasmic localization and progressive motoneuron loss although, mice did not recapitulate ALS end stage (Scekic-Zahirovic *et al.*, 2016). However, another knockout model for *FUS* showed no sign of motor defects, mice displayed phenotypes like hyperactivity but no ALS feature even at adult stage (Kino *et al.*, 2015). Models with mutations altering the nuclear localization sequence have also been generated to assess the role of the mislocalization. Most mutant mouse for FUS displayed motoneuron loss, muscle denervation and motor deficits (Devoy *et al.*, 2017; Picchiarelli *et al.*, 2019; Sharma *et al.*,

2016). Recently, Devoy et al have engineered mouse models replacing the mouse sequence by the human sequence for Sod1, Tardpb and Fus (Devoy *et al.*, 2021). These changes did not trigger major changes in expression and seem to be useful for future therapeutic treatment development.

Mouse also usually need an overexpression of the mutant genes to recapitulate relevant phenotypes. Although mice model has brought great insights about the disease, it allows to model only genetics forms of ALS which represents 10 percent of all ALS cases. Despite being the reference for pre-clinical trial, this model has failed to give results for new clinical treatments.

1.5.3 Drosophila

Drosophila is used to model ALS as it has the advantage to have many genetics tools to develop models. These tools allow the creation of model having special genetic mutation or models that have tissue specific expression of proteins like the UAS-Gal4 system. To study ALS, Drosophila has several tests like the crawling and climbing assays, or hatching rate to assess motricity, lifespan can be assessed as well, and even electrophysiological recordings can be done. In addition, flies have a several ways to be administered with drugs, it can be by feeing, by inhalation or directly injected.

Flies seem to recapitulate the main features to study ALS. Indeed, around ten ALS model have been generated for SOD1 and all of these models have been showing motor defects and reduced lifespan (Held *et al.*, 2019; Mockett *et al.*, 2003; Sahin *et al.*, 2017). *dsod1*^{G85R} also showed a decrease in the number of NMJ and defects in electrophysiology tests. Although, human mutation G93A, the most widely used in mice, fail to show any decrease in the SOD1 activity (Yim *et al.*, 1996).

Overexpressing flies with either a wilt type *FUS* and mutant *FUS* seem to also show strong phenotype with a high-rate lethality and reduced lifespan (Bogaert *et al.*, 2018; Lanson *et al.*, 2011; Miguel *et al.*, 2012; Wang *et al.*, 2011; Xia *et al.*, 2012). The only ortholog mutant for *FUS* in Drosophila being *caz*, a study has been assessing the effects of a mutation in this gene and has found that *caz* mutants show motor defects and decreased lifespan (Frickenhaus *et al.*, 2015; Wang *et al.*, 2011; Xia *et al.*, 2012). Overexpressing the wild type *FUS* has successfully rescued the phenotype in these mutants while the mutant forms of the gene could not show an effect of these mutations in flies.

Mutant flies have been created for *TBPH*, TDP-43 ortholog and mutants have been displaying reduced lifespan, lethality and motor defects (Diaper *et al.*, 2013; Feiguin *et al.*, 2009; Fiesel *et al.*, 2010; Lin *et al.*, 2011b). However, these models do not show the same results about NMJ branching. Some models show a decrease of NMJ number and axonal branch while other did not show any difference.

Drosophila can be used to model and study genetic mutations and even try drug screening but this model has limitations such as the anatomical differences, immune system that lacks the adaptive part or the lack of orthologs for ALS key genes.

1.5.4 *C. elegans*

C. elegans is a small nematode that has a lot of genetics tool available to generate mutant models. Around 35 percent of human genes have an ortholog in *C. elegans* and most of these genes have around 30 percent of homology (Therrien *et al.*, 2014). This makes the nematode a very useful model since it has a fast life cycle, it can be used for high throughput drug screening. It has been reported, using different transgenic model, that most ALS related cellular stress pathways were conserved in the worm (Caldwell *et al.*, 2020). *SOD1* mutant models have been created and showed that mutations in this gene prevents cellular response to oxidative stress, thus, promotes protein aggregation (Oeda *et al.*, 2001). *SOD1*^{G83R} expression in worm nervous system provoked motor phenotypes and synaptic dysfunction (Wang *et al.*, 2009). Recently, metformin was used to activate autophagy on mutant expressing worm for *SOD1*, and an increased lifespan with an improved motor behaviour were observed (Xu *et al.*, 2022). Worms expressing a mutant or truncated *FUS* gene showed motor phenotypes and synaptic dysfunction (Murakami *et al.*, 2012; Vaccaro *et al.*, 2012). In 2010, a first mutant expressing a human mutant version of TDP-43 showed impaired motor phenotypes (Ash *et al.*, 2010). These results were corroborated by two other studies that found expression of human mutant of TDP-43 in worm nervous system leads to impaired motor behaviour, synaptic dysfunction and protein aggregation (Liachko *et al.*, 2010; Zhang *et al.*, 2012). As a drug discovery tool, *C. elegans* was used to screen and find α-metil-α-phenylsuccinimide as a potential neuroprotective treatment in TDP-43 mutants (Wong *et al.*, 2018).

1.5.5 Zebrafish

Zebrafish is a simple vertebrate model for ALS. Just like the other model organisms, its genome is known and genetic tools have been developed to generate mutants. Its transparent development makes it very easy to study and image. Main organs, tissues and systems are fully functional at two days post fertilization (dpf) including the motor system (Kimmel *et al.*, 1995). Indeed, axogenesis starts at 20 hours post fertilization (hpf) and by two dpf, axons have reached their target muscles (Lewis *et al.*, 2003; Myers *et al.*, 1986). This fast development makes the Zebrafish a great model to study ALS without having to wait too long to observe a degeneration. Despite being smaller, the overall organization of the nervous system is quite conserved between human and zebrafish. Cell types and neuron structure are similar (Babin *et al.*, 2014) plus, motor and sensory systems as well as neurotransmitters are conserved.

84 percent of diseases associated genes in human have a zebrafish ortholog (Howe *et al.*, 2013) which is why it can be used to model and study mutations. Genome editing tools such as CRISPR-Cas9 or the Tol-2 system can be used to create stable lines. Historically, antisense morpholinos oligonucleotides (AMOs) were the most widely used tool to study a LOF. Although AMOs expression is transitory so it had to be studied in the first days of the fishes which may not always be relevant for a degenerative disease. Moreover, AMOs had a high off target effect and can activate p53-dependent apoptosis which can bias the analysis (Kok *et al.*, 2015). In 2004, Tol2 system was used in Zebrafish to stably integrate a sequence in the genome (Kawakami *et al.*, 2004). The Tol2 element codes for an active transposase that can integrate the co-injected construct. For example, a stable Zebrafish model has been created to model spinal muscular atrophy using micro-RNAs (Giacomotto *et al.*, 2015). Currently, CRISPR-Cas9 is the most widely used system to generate Zebrafish mutants to model genetic diseases. It has a reduced off target effect and is stable (Hruscha *et al.*, 2013). It only requires a guide RNA to target the location of the mutation and the Cas9 being a nuclease, can cut the DNA inducing a non-homologous end-joining (NHEJ) and a mutation.

Lastly, Zebrafish is a good model to test potential therapeutics in a high throughput manner since it is permeable to small molecule when put directly in the water (Zon *et al.*, 2005). Its small size and large clutches of embryos allow to test on a high quantity of fishes at the same time and with tests as easily assessed as the motor activity, fishes have a high potential to help drug discovery.

Zebrafish allows rapid and easy modelling of human diseases although like all model organisms, it has limitations such as differences in the immune system or even in the motor system.

To model ALS, Zebrafish has been showing a great conservation of the main feature of the disease such as motor dysfunction resulting from a motoneuron degeneration. Indeed, injection of 3 different mRNAs encoding three different mutations, G93A, G37R, A4V, led to motor axonopathy (Lemmens *et al.*, 2007). In addition, a fish line overexpressing mutant *SOD1* G93R showed NMJ defects, motoneuron loss, muscle atrophy, paralysis and premature death (Ramesh *et al.*, 2010). Accordingly, a stable line expressing mutant *SOD1* G93A have been generated using the Tol2 system as well to assess late effect of the mutation (Sakowski *et al.*, 2012). Fish showed NMJ defects at 20 weeks of age followed by a motoneuron loss. Knocking down *FUS* using AMO injection led to defective motor function in fish (Armstrong *et al.*, 2013b). Both knock down and overexpression of a mutant *FUS* led to NJM synaptic transmission defects and mutant *FUS* triggered a hyperexcitability in the motoneurons. Mutant *TARDBP* expressing three different mutations, A315T, G348C, A382T were generated and showed motoneuron sensitivity and decreased motor activity (Kabashi *et al.*, 2011a). Knock down of *tardbp* ortholog in the zebrafish genome also led to motoneuron axonal defects and altered swimming behaviour. Another mutant TDP43 zebrafish was created and displayed motor dysfunction and that primary motoneurons were hyperexcitable (Armstrong *et al.*, 2013a). Synaptic activity at the NMJ in those fish was impaired and led to a denervation of the muscles. This fish line expressing *tardbp*G348C was then used for a drug screen that could identify Pimozide as a novel potential therapeutic approach to increase synaptic transmission at the NMJ (Patten *et al.*, 2017). Both GOF and LOF *C9orf72* model in zebrafish have also been created and will be describe in section 1.7.

1.6 *C9orf72* in ALS

The hexanucleotide repeat expansion (HRE) of GGGGCC in the first intronic region of the gene *C9orf72* leads to three pathological mechanisms: two toxic GOF including RNA foci accumulation and dipeptide repeats aggregation; and a LOF of the gene leading to haploinsufficiency. The three mechanisms and their effects will be described in this section.

1.6.1 RNA foci

The HRE (GGGGCC) in the first intronic part of the gene has several implications. The first one is a toxic GOF. It is due to the transcription of the HRE leading to toxic accumulation of RNA in the nuclei of the cells, mostly at the edge of the nuclear membrane but they can be occasionally found in the cytoplasm as well (Cooper-Knock *et al.*, 2014; Donnelly *et al.*, 2013; Mizielinska *et al.*, 2013). RNA foci can by produced by both sense and antisense transcription of the HRE and

have been found in patient brains, and more precisely in the nuclei of neurons from the frontal and motor cortex, hippocampus, cerebellum and spinal cord (Mizielinska *et al.*, 2013) (Cooper-Knock *et al.*, 2014; Lagier-Tourenne *et al.*, 2013). RNA foci can also be found at lower levels in glial cells such as astrocytes, oligodendrocytes and microglia (Mizielinska *et al.*, 2013). The toxicity emerging from these RNA foci comes from the fact that they can form secondary structures as hairpins and G-quadruplex for the sense RNA, i-motif and quadruplex from antisense RNA (Fratta *et al.*, 2012; Kovanda *et al.*, 2015; Reddy *et al.*, 2013; Zamiri *et al.*, 2015). These structures favour the sequestration of RNA binding proteins (RBPs) such as hnRNP A1, hnRNP A3, hnRNP H, ALRYREF, ADAR2 of pur-α leading to their mislocalization and altering their functions, gene stability, regulation, splicing or RNA translation for instance (Cooper-Knock *et al.*, 2014; Donnelly *et al.*, 2013; Haeusler *et al.*, 2014; Kharel *et al.*, 2020; Lee *et al.*, 2013; Sareen *et al.*, 2013; Xu *et al.*, 2013). From an intracellular point of view, RNA foci have been localized on the edge of the nuclear membrane which can explain the nucleo-cytoplasmic transport defects (Mizielinska et al, 2013; Zhang et al, 2015). Foci have also been found to be associated with the nucleolus, a membraneless structure where ribosomal RNA transcription takes place, thus having a role in the RNA dysregulation (Aladesuyi Arogundade *et al.*, 2019).

RNA foci are a hallmark of the C9orf72 pathology although their toxicity has been discussed, indeed, two studies have found opposite conclusions about RNA foci toxicity and clinical features, one claiming that more antisense RNA foci found in the frontal cortex would correlate with an earlier age of onset while the second one states that more antisense RNA foci in middle frontal gyrus neurones would correlate with a later age of onset (DeJesus-Hernandez *et al.*, 2017; Mizielinska *et al.*, 2013). While no conclusion can properly be drawn, it seems like brain RNA foci level cannot be correlated to clinical features in patients.

1.6.2 Dipeptide repeats proteins

Non-canonical way, repeat-associated non-ATG (RAN) translation leads to the expression of five dipeptides: proline-arginine (PR), proline-alanine (PA), glycine-proline (GP), glycine-arginine (GR), glycine-alanine (GA). These dipeptides accumulate and form inclusion in cells, these inclusions are p62 positive and TDP-43 negative (Ash *et al.*, 2013) (Gendron *et al.*, 2013) (Mackenzie *et al.*, 2013) (Mackenzie *et al.*, 2014) (Mori *et al.*, 2013b) (Zu *et al.*, 2013). DPR have been reported to be in neurons and glial cells (Schludi *et al.*, 2015) (Mori *et al.*, 2013b) (Mann *et al.*, 2013) (Ash *et al.*, 2013). Regarding brain regions, DPR have been found in the hippocampus, in frontal and motor cortex, basal ganglia and cerebellum of patients (Al-Sarraj *et al.*, 2011;

Cooper-Knock *et al.*, 2012; Hsiung *et al.*, 2012; Mahoney *et al.*, 2012; Troakes *et al.*, 2012). Although, DPR are found in very low quantities in the spinal cord and motoneurons (Gomez-Deza *et al.*, 2015; Schipper *et al.*, 2016).

While DPR toxicity has been extensively reported in cells and animal models, each of the five DPR show different contribution to the disease mechanism (Kramer *et al.*, 2018; Lee *et al.*, 2016; Swaminathan *et al.*, 2018). Arginine rich DPR, poly-GR and poly-PR are the most toxic DPRs due to their charge and polarity. They promote cell death when added to the cell media starting 10μM concentration (Kwon *et al.*, 2012). Drosophila has showed a high toxicity of these DPR in their eyes, neurons, glial cell and motoneurons whereas, poly-GA, poly-PA and poly-PR did not show this much toxicity in those tissues (Freibaum *et al.*, 2015; Lee *et al.*, 2016; Mizielinska *et al.*, 2014; Wen *et al.*, 2014). In a zebrafish model, poly-GR expression impairs motor functions and reduces axon length and, in another model, poly-GR promotes cellular death in fish brains and lead to abnormal motoneuron morphology (Riemslagh *et al.*, 2021b; Swaminathan *et al.*, 2018). A mouse model expressing poly-GR100 has developed brain atrophy, neurodegeneration as well as motor and memory deficits. These mice also showed that poly-GR interacts with ribosomal subunits, translation factors and stress granules associated proteins to impair ribosomal activity, protein translation and stress granules dynamics (Zhang *et al.*, 2018d). In cells, poly-GR accelerates and promotes TDP-43 aggregation, moreover, poly-GR could sequester TDP-43 in the cytoplasm. In addition, a mouse model expressing a GFP-$(GR)_{200}$ showed the TDP-43 positive inclusions previously found in cells and defects in the nucleo-cytoplasmic defect, a key feature of ALS. This transport would be disrupted because of the sequestration of nucleoporins and importins by poly-GR (Cook *et al.*, 2020). Indeed, another study has documented an interaction between arginine rich DPR, GR and PR with importin b, disrupting nucleocytoplasmic transport (Hayes *et al.*, 2020). Nevertheless, a study has failed to link poly-GR and poly-PR to nucleo-cytoplasmic transport defects (Vanneste *et al.*, 2019). This disruption would be a consequence of another defective pathway, via stress granules formation or protein such as TDP-43 aggregation for example. Although a recent study suggest that poly-GR, poly-PR and poly-GA provoke a fragmentation of the nuclear membrane thus, nuclear structure damage (Ryan *et al.*, 2022). This study also adds that it would match with the results of another study that links poly-PR expression in mice with fragmentation of lamin-B1 and lamin-A/C leading to nuclear membrane damages (Zhang *et al.*, 2019b).

Poly-GR is found in higher concentration than poly-PR which explains its higher toxicity (Lee *et al.*, 2016; Wen *et al.*, 2014). However, poly-PR has been reported to bind ADAR1 and 2 to inhibits

their editing activity which leads to the previously described lack of edition of GluA2 and excitotoxicity (Suzuki *et al.*, 2021). Although, another report claims that patients harboring the *C9orf72* mutation did not show any lack of edition at the Q/R site in the GluA2 protein sequence (Selvaraj *et al.*, 2018). Still on the hyperexcitability side, Jo and colleagues have reported that mice cortical neurons showed a hyperexcitability after exposing them to poly-PR dipeptides (Jo *et al.*, 2022). They explain this phenomenon by reporting that poly-PR can bind sodium channel and lead to a persistent sodium current.

The other moderately toxic DPR is poly-GA, it is the most widely found in inclusions from patient brain and spine (Mackenzie *et al.*, 2015; Mackenzie *et al.*, 2014; May *et al.*, 2014). Poly-GA has been found to be prone to aggregation and giving filamentous forms. These aggregates have a role in the pathological mechanisms by disrupting nucleo-cytoplasmic transport, the ubiquitin proteasome system, and increasing endoplasmic reticulum stress (Yamakawa *et al.*, 2015; Zhang *et al.*, 2016; Zhang *et al.*, 2014). Poly-GA toxicity has also been explained by its ability to sequester Unc119 which is involved in axonal maintenance (May *et al.*, 2014). A study using mice model has found that expressing high level of poly-GA, mice developed motor and cognitive defects, these impairments are along neuronal toxicity, cerebellar atrophy and astrogliosis (Chew *et al.*, 2015; Zhang *et al.*, 2016).

The last two dipeptides, poly-PA and poly-GP, on the contrary, do not seem to have any toxic effect. They are not found to interact with other proteins (Lee *et al.*, 2016).

1.6.3 Haploinsufficency

The level of *C9ORF72* mRNA is decreased by 38 to 50 percent in ALS patient frontal cortex samples (DeJesus-Hernandez *et al.*, 2011; Gijselinck *et al.*, 2012). Moreover, V1 and V2 *C9ORF72* transcripts were found to be decreased as well in patient frontal cortex and cerebellum (Belzil *et al.*, 2013; Fratta *et al.*, 2013; Rizzu *et al.*, 2016; van Blitterswijk *et al.*, 2015; Waite *et al.*, 2014) and also in spinal cord and motor cortex samples (Donnelly *et al.*, 2013). While GOF mechanisms were extensively studied, LOF role in ALS physiopathology was less so studied. *C9orf72* LOF mechanism has been controverted for two reasons, the first one being that a homozygosity of the mutation does not increase the phenotype (Cooper-Knock *et al.*, 2013) although there are only two cases so far to be reported (Fratta *et al.*, 2013). The second reason is that a LOF mutation has only been reported once in *C9orf72* gene. This mutation leading to a c.601-2A>G change creating a stop codon and a truncated protein has been found on one unique

patient in China but except this one patient, only the HRE in known to have a link with ALS (Harms *et al.*, 2013; Liu *et al.*, 2016a).

In patient derived motoneurons (iMNs) from iPS cells, low levels of *C9orf72* mRNA were observed (Shi *et al.*, 2018). These iMNs could recapitulate ALS related phenotype and that some of these phenotypes were rescued upon reexpression of *C9orf72*. Lastly, the same study shows that decreasing *C9orf72* expression in control iMNs reduces the survival of these cells to the same level as patients iMNs.

On the other hand, epigenetics has a role as well. Indeed, a third of the patients have been harbouring a hypermethylation in the promoter region of *C9ORF72* (Liu *et al.*, 2014a). In addition to the promoter, the expansion itself can be hypermethylated and three studies have correlated it to a shorter lifespan for patient and a more aggressive disease course (Gijselinck *et al.*, 2016; Jackson *et al.*, 2020; Liu *et al.*, 2014a) while two other studies have been correlating the hypermethylation profile with an increased survival (Russ *et al.*, 2015). This latter finding has been proposed as a protection mechanism to avoid the formation of toxic RNA foci or dipeptides (McMillan *et al.*, 2015).

On the protein level, C9orf72 levels show a 25 percent decrease of expression (Saberi *et al.*, 2018; Waite *et al.*, 2014; Xiao *et al.*, 2015) which can be considered as a mild decrease but studying disease relevant cell type specific level of decrease could definitely bring new insights into this phenomenon.

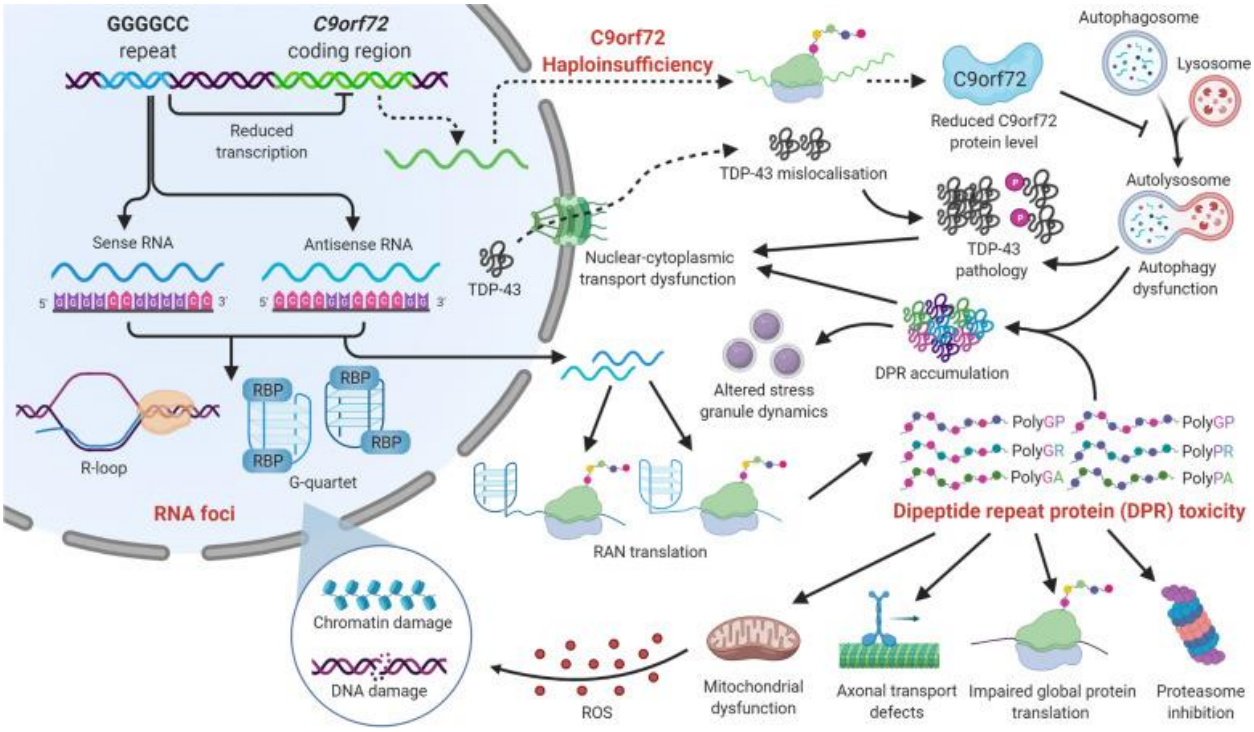

Figure 1-5: C9orf72 pathological mechanisms overview.

The hexanucleotide (GGGGCC) repeat expansion leads to a decrease expression of *C9orf72* and a haploinsufficiency, bidirectional transcription of the repeats leads to RNA foci formation that can sequester proteins. And last, RAN translation leads to dipeptide repeat production that can disturb many cellular pathways including axonal transport, autophagy, proteasome function for example. Adapted from (Masrori *et al.*, 2022). (Authorized by CC BY 4.0 licence, Creative Common).

1.6.4 Role of C9orf72

C9orf72 protein is expressed in both cell nucleus and cytoplasm (Frick et al, 2018). Cytoplasmic distribution correlates with vesicular distribution matching endosomes, lysosomes, autophagosomes (Farg *et al.*, 2014; Maharjan *et al.*, 2017; Shi *et al.*, 2018). It has also been found in the pre-synaptic end of neurons (Frick *et al.*, 2018).

C9orf72 is found has a complex with Smith-Magenis Chromosome Region 8 (SMCR8) and WD40-repeat containing protein 41 (WDR41) (Amick *et al.*, 2016; Sellier *et al.*, 2016; Sullivan *et al.*, 2016; Ugolino *et al.*, 2016; Webster *et al.*, 2016; Yang *et al.*, 2016). The protein sequence of C9orf72 shows the presence of a Differentially Expressed in Normal and Neoplasia (DENN) domain. DENN domains have been found to be Guanosine TriPhosphate (GTP)-Guanosine DiPhosphate (GDP) Exchange Factors (GEF). These GEFs oversee activation and deactivation of Rab GTPases which are key regulators of membrane trafficking with a great role in autophagy.

That is why C9orf72 role has been studied towards autophagy pathway. Indeed, C9orf72 in complex with SMCR8 and WDR41 interacts with Rab39b very early in the initiation of autophagy, during the formation of the autophagosome (Nassif *et al.*, 2017; Sellier *et al.*, 2016; Yang *et al.*, 2016). Via Rab1 and Rab5 regulation, C9orf72 has a role in ULK1 complex trafficking during autophagy initiation (Amick *et al.*, 2017; Sullivan *et al.*, 2016; Webster *et al.*, 2016). By interacting with Rab7 and Rab11, C9orf72 would also have a role in autophagosome closure and maturation (Farg *et al.*, 2014). And later in the autophagy pathway, regulation of Rab8a would make C9orf72 involved in fusion between the autophagosome and lysosome (Amick *et al.*, 2017). That explains how a C9orf72 LOF would lead to autophagic defects by a disruption of the autophagosome/lysosome homeostasis leading to their accumulation (Amick *et al.*, 2017; Amick *et al.*, 2016; Corrionero *et al.*, 2018).

C9orf72 has also been identified to be a GTPase activating protein (GAP) for ADP ribosylation factor (ARF) proteins. Indeed, the C9orf72-SMCR8-WDR41 complex has been reported to be a GAP for ARF1 and ARF6 (Sivadasan *et al.*, 2016; Su *et al.*, 2021; Su *et al.*, 2020). ARF protein are involved in vesicular trafficking by interacting with coating complexes (D'Souza-Schorey *et al.*, 2006). Thus, C9orf72 LOF can contribute to the disease due to a lack of activation of ARF proteins and by extension, a lack of vesicular transport, signalling and autophagy (Su *et al.*, 2021).

C9orf72 plays a role in several cellular processes. For instance, it has been found to be involved in stress granule formation and degradation (Chitiprolu *et al.*, 2018; Maharjan *et al.*, 2017). C9orf72 seems to be recruited upon stress signals such as endoplasmic reticulum stress or a heat shock in mice neurons. In this study, it is also showed that C9orf72 is necessary to the stress granule formation since a LOF prohibits stress granule assembly.

As mentioned previously, C9orf72 is found in the pre-synaptic part of neurons (Frick *et al.*, 2018). Another study has localized C9orf72 in both pre- and post-synaptic compartments (Xiao *et al.*, 2019). This latter study has reported that C9orf72 would interact with a complex containing Rab3a, leading us to think that C9orf72 has a role at the synaptic junction (Xiao *et al.*, 2019). Although, changes in C9orf72 expression did not alter the level of Rab3a in the presynaptic terminal. Besides, on the post-synaptic compartment, C9orf72 would have a role in glutamatergic receptor recycling via Rab39b regulation. Overall, C9orf72 has been identified at the synapse but its role remains unclear.

Axonal growth would also be regulated by C9orf72 since primary mouse embryonic motor neurons overexpressing C9orf72 show longer axon and a bigger growth cone size. And the opposite effect has been showed as well (Sivadasan *et al.*, 2016).

The last role described here for C9orf72 is towards immune regulation. *C9orf72* transcripts are found in human and mice myeloid cells (Rizzu *et al.*, 2016). In addition, mice LOF mutant for C9orf72 do not show motor phenotype but immune abnormalities and diseases (Atanasio *et al.*, 2016; Burberry *et al.*, 2016; Koppers *et al.*, 2015; O'Rourke *et al.*, 2016; Sudria-Lopez *et al.*, 2016; Sullivan *et al.*, 2016; Ugolino *et al.*, 2016). Those mice showed splenomegaly and enlarged lymph nodes accompanied by a shortened lifespan. A genetic screen on human cells has reported that C9orf72 would interact with FIS1, which is involved in inflammatory cascade (Chai *et al.*, 2020).

1.7 Available models to study C9orf72

The different controversies and points of view described in the previous part emphasize the need to model C9orf72 pathology in various model and using different approaches. Modelling only GOF can bring new insights in the disrupted mechanisms while LOF model can bring new information about the endogenous role of C9orf72 and understand how the expansion leads to pathological defects.

1.7.1 Gain of function models

Studying GOF for C9orf72 can be done using different approaches since two mechanisms are involved. In simple organisms such as Drosophila, repeats expressing models have been used to study the toxic GOF. Given that Drosophila does not have an ortholog for C9orf72, the LOF contribution cannot be taken in consideration. Several studies have demonstrated that expressing RNA repeats in Drosophila lead to high neuronal toxicity and reduced lifespan indicating that in this model, GOF alone is sufficient to engender neurodegeneration (Freibaum *et al.*, 2015; Mizielinska *et al.*, 2014; Moens *et al.*, 2018; Xu *et al.*, 2013). Although, it seems like the toxicity in one model would be more explained by the arginine-rich dipeptide contribution, but it does not exclude RNA foci contribution in toxicity (Mizielinska *et al.*, 2014). It is supported by the fact that treatment with cycloheximide that inhibits protein synthesis partially rescues the phenotypes observed in the flies. This study also shows that the length of the repeats used in the flies have an effect of the phenotype, indeed, a longer repeat size lead to more severe phenotype. This latter finding is consistent with other studies using other model organisms such as Zebrafish (Lee *et al.*, 2013) or mice (Herranz-Martin *et al.*, 2017). In 2018, a study expressing RNA foci only in flies have not been able to show any type of neurodegeneration, supporting again the fact that toxicity is due to the dipeptide more than the RNA (Moens *et al.*, 2018). Another fly study using repeats leading to RNA foci and dipeptides have showed that it led to a decreased number of

synaptic boutons and altered motricity (Freibaum *et al.*, 2015). Flies have also been greatly used to investigate the role of each DRP in toxicity (Boeynaems *et al.*, 2016; Freibaum *et al.*, 2015; Mizielinska *et al.*, 2014; Moens *et al.*, 2018; Solomon *et al.*, 2018; West *et al.*, 2020; Xu *et al.*, 2018). Overall, studies show that poly-GA, poly-GR and poly-PR have the higher toxicity and show the stronger phenotypes but a new study has showed that co-expressing poly-PR and poly-GR in the fly can lead to an even stronger phenotype, seizures accompanied by motor defects (West *et al.*, 2020). These seizures are a sign that these DPR may have an implication about neuronal hyperexcitability. Recently, a Drosophila model for C9orf72 DPR has been linking axonal transport defects to arginine rich DPR (Fumagalli *et al.*, 2021). This finding has been verified in patient derived motoneurons and mouse model which has also confirmed that arginine-rich DPR interact with microtubules and motor proteins. Besides, a recent study investigated to role of glia in ALS in a GOF fly model and found that a C9orf72 GOF in glia lead to motor defects and muscle atrophy (Farrugia *et al.*, 2022).

In Zebrafish, GOF can easily be studied by injection of repeats mRNA and in 2013, a team has been studying a model expressing 8, 38 or 72 repeats (Lee *et al.*, 2013). They showed that these repeats were forming the secondary structure like G-quadruplex found in cell models and that the toxicity was length dependant in the fishes. In addition, they showed that those secondary structures have the ability to sequester RNA binding proteins lead to a defect in their function. Lastly, they have showed that the fishes expressing the longest repeats construct were showing a higher apoptosis cell rate leading to think that repeat injection is enough to trigger cell death in fish. In the same way, a 2018 study has been injecting three types of constructs in fish, 35, 70 and 90 repeats long constructs were tested (Swinnen *et al.*, 2018). In opposition to the previous studies, this study found no difference in the phenotype between the different construct length presuming that the toxicity comes from a threshold effect starting around 30 repeats expression. Zebrafish expressing the repeats showed motor axon abnormalities including a reduced axonal growth and aberrant branching. It was showed as well that by binding Pur-alpha, a protein involved in autophagy, RNA foci were leading to a toxicity not depending in the DPR. The phenotype was rescued by the expression of pur-alpha underlining the specificity of the mechanism. DPR toxicity was also studied using a 50 repeat long encoding DPR with an ATG codon. Again, arginine rich dipeptides showed the higher toxicity leading to motor axon abnormalities. To this point, both Zebrafish models led to interesting findings although, they both used a transitory expression which prevent from studying the disease at later stages.

In 2018, a new Zebrafish model has been created stably expressing an 89 long repeats construct (Shaw *et al.*, 2018). Fishes exhibited RNA foci and DPR from both sense and antisense directions in muscles and nervous system cells. Motor defects appeared after five days and adults could be studied to investigate later stage of repeats expression. Beside an early death observation, muscle atrophy, cognitive impairment and loss of motoneurons could characterized the adult stage leading us to think that the model would be suitable to study the disease further and use for drug screen. Two other studies have investigated the role of DPR toxicity in Zebrafish (Ohki *et al.*, 2017; Swaminathan *et al.*, 2018). The first one investigated the role of poly-GA and showed that this single dipeptide can lead to degeneration and be used as a model for drug screens (Ohki *et al.*, 2017) while the second study used poly-GA, poly-GR, poly-PA and poly-PR (Swaminathan *et al.*, 2018). It was showed that those four dipeptides expression were leading to a loss of motor activity in fish. More precisely, poly-GR seems to lead to a defect in motoneuron growth explaining how the motor activity can be affected.

In mice, modelling GOF mechanisms has been done using different tools. One of the most used is the C9-BAC (Bacterial Artificial Chromosome) model using then a bacterial chromosome to express the repeats (Jiang *et al.*, 2016; Mordes *et al.*, 2020; O'Rourke *et al.*, 2016; Pattamatta *et al.*, 2021). C9 BAC containing 500 repeats (C9-500) mouse model showed motor phenotype like paralysis, motoneuron loss, NMJ denervation and muscle atrophy prior to death (Liu *et al.*, 2016b). However, a team reported that a mouse model expressing a BAC carrying the full C9orf72 human hexanucleotide repeat expansion did not show any denervation or NMJ loss even with RNA foci found in the nervous system of those mice (O'Rourke *et al.*, 2016). No motor phenotypes were reported in those mice, even at advance life stages. This study has been supported by two other studies reporting similar conclusions with no motor phenotypes observed in C9-500 BAC carrying mice (Mordes *et al.*, 2020; Peters *et al.*, 2015). A more recent study reports that C9orf72 effect is length dependant (Pattamatta *et al.*, 2021). Indeed, they found that a longer HRE triggers the disease earlier with a more severe phenotype and increase levels of RNA foci and dipeptide aggregates.

Another approach for GOF in mice is using adeno-associated virus (AAV) to deliver repeats. Herranz-Martin and colleagues reported that 102 repeats carried by an AAV in mice would trigger NJM defects and behavioural impairments (Herranz-Martin *et al.*, 2017). Lastly, HRE pathology has been modeled using a doxycycline inducible system to express 36 repeats and this model has shown motor defects with muscle atrophy although this model showed no RNA foci even after 24 weeks of treatment (Riemslagh *et al.*, 2021a).

A rat knockin model using CRISPR-Cas9 to stably integrate 80 (GGGGCC) repetitions in the first intron of the C9orf72 ortholog in rat genome displayed motor deficits and partial paralysis as well as decreased number of spinal motoneurons (Dong *et al.*, 2020). Abnormalities in the immune system was also reported, similarly to the findings in mice. The presence of RNA foci and DPR was not notified, although, interestingly, an inhibition of C9orf72 protein expression was reported suggesting that the HRE is indeed responsible for the LOF and that the LOF mechanism has a strong contribution in ALS pathology.

1.7.2 Loss of function models

LOF model have been showing various phenotypes depending on the model organism used. In simple genetic model organisms like C. elegans or the zebrafish, C9orf72 LOF leads to motoneuron degeneration. The C. elegans model with a null mutation in the *C9orf72* orthologue showed age dependant motility defects and paralysis accompanied with the degeneration of the GABAergic motoneurons (Therrien *et al.*, 2013). In the same way, a LOF model for *C9orf72* using morpholinos has been done in zebrafish (Ciura *et al.*, 2013). This model has shown a decrease of both spontaneous and evoked motor activity. At the neuronal level, fish larvae injected with morpholinos targeting *C9orf72* showed a disturbed arborization, shortened axons and a motoneuron axonopathy (Ciura *et al.*, 2013). Although, morpholinos have a transitory expression, these defects could only be assessed at early stage of the fish development and no end stage could be investigated.

Another team created a zebrafish model having two different truncated versions of C9orf72 protein, one containing only the upstream region of the DENN domain found in C9orf72, and the other one containing only the central region of the DENN domain (Yeh *et al.*, 2018). Both constructs led to defective neuronal network and decreased response to stimuli indicating a dominant negative effect of the mutations. It demonstrates as well that the DENN domain in C9orf72 protein sequence has a key role in GTPase activity which is fundamental in vesicle formation and transport in cells. Fishes showed defects in axon formation in the hindbrain and defects in motor activity bringing us to think that C9orf72 has a key role in neuron function and synapse formation.

In mice, knock down of *C9orf72* using antisense oligonucleotides (ASOs) has not led to any behavioural defect of pathological symptoms (Lagier-Tourenne *et al.*, 2013) and other knock-out models have been showing neuroinflammation and myeloid dysregulation albeit no motor phenotypes were observed (Burberry *et al.*, 2016; Jiang *et al.*, 2016; Koppers *et al.*, 2015;

O'Rourke *et al.*, 2016; Sudria-Lopez *et al.*, 2016). What has been seen in these mice models is a lack of successful decrease at the protein level while the mRNA level would indicate a 50 to 70 percent decrease (Atanasio *et al.*, 2016; Burberry *et al.*, 2016; Lagier-Tourenne *et al.*, 2013). In addition, no TDP-43 or p62 pathology were observed. In 2019, Shao and colleagues demonstrated a dose dependent effect of *C9orf72* LOF in mice (Shao *et al.*, 2019). By crossing C9-BAC carrying mice with *C9orf72* heterozygous mutant for a knockout, they could generate a model combining GOF and LOF which gave motor deficits, indicating that LOF of *C9orf72* is a contributor to ALS (Shao *et al.*, 2019).

A rat model with an ablation of *C9orf72* expression using CRISPR-Cas9 to excise a part of the gene leading to a premature translation termination showed motor deficits and a loss of motoneurons when treated with an inducer of excitotoxicity, kainic acid (Dong *et al.*, 2021). This suggests that *C9orf72* haploinsufficiency engenders a higher sensitivity to stressors to induce ALS. Rats also displayed a severe golgi complex fragmentation and altered distribution of lysosomes, endosomes and recycling endosomes in the spinal cord. Lastly, an abnormal immune phenotype was reported with a splenomegaly and a lymphoadenopathy similar to mice phenotypes (Dong *et al.*, 2021).

1.8 Hypothesis and aims

Overall, ALS is a highly heterogenous disease, clinically and biologically. The lack of curative treatment reflects the difficulty to target a pathway to slow down disease progression or revert it. Various cellular defects have a role in the disease, leading to motoneuron death. More particularly, synaptic defects which appear early in the disease, lead to the loss of motoneurons, that is why it is important to find a way to preserve the neuromuscular synapses.

C9orf72 discovery and the lack of knowledge about its role led to many hypotheses and investigations. Both toxic RNA foci and dipeptide repeats aggregation are extensively studied while *C9orf72* haploinsufficiency has been proved to be an important part of the *C9orf72* pathology and remains less explored. Our overall goal is to understand the function of C9orf72 in the CNS and the contribution of its loss towards ALS development.

Bioinformatics analyses predict the presence of a DENN domain in C9orf72 protein sequence known to be regulator of the small GTPases of the Rabs family proteins (Levine *et al.*, 2013). Rabs-GTPases are key regulators of the formation, trafficking and fusion of vesicles implicated in autophagy and synaptic transmission (Corbier *et al.*, 2017; Geppert *et al.*, 1997; Shimizu *et al.*, 2003; Star *et al.*, 2005). Indeed, several studies reported a role of C9orf72 as a potential GEF for Rabs in vesicle trafficking. However, these studies are primarily focused on endocytic and autophagic vesicles, implicating C9orf72 function in endosomal trafficking and autophagy while the synaptic compartment remains less investigated. Synaptic transmission requires several Rabs activation such as Rab3, Rab39 or Rab27 for example. Additionally, Xiao et al recently reported that C9orf72 is expressed pre—and post-synaptically and Frick et al reported that C9orf72 is localized in the presynaptic compartment as well (Frick *et al.*, 2018; Xiao *et al.*, 2019).

C9orf72 physiological function at the synapse, and particularly pre-synaptically remains largely unexplored. A better understanding of its synaptic function is essential to uncover *C9orf72* LOF contribution to ALS pathogenesis and uncover therapeutic targets. I hypothesized that *C9orf72* LOF is a major pathological mechanism in ALS pathogenesis leading to synaptic defects at the NMJ that would appear before the motoneuron death.

The objective of my PhD project was to first develop a *C9orf72* ALS LOF model in zebrafish and use the model to understand function of C9orf72 particularly at the NMJ.

My specific aims during the PhD are:

Aim 1: To generate and characterize a knockdown zebrafish model for *C9orf72*.

Aim 2: To investigate the changes in synaptic morphology and properties at the NMJ in the zebrafish *C9orf72* model.

Aim3: To use the zebrafish *C9orf72* model to test candidate molecules against ALS-like phenotypes.

Experimental approach and findings of the aims 1 and 2 are described in Chapter 2 and forms part of a published article (Butti et al, 2021, Communication biology). Experimental approach and findings of aim 3 are described in Chapter 3 and will form part of a paper in preparation.

2 REDUCED C9ORF72 FUNCTION LEADS TO DEFECTIVE SYNAPTIC VESICLE RELEASE AND NEUROMUSCULAR DYSFUNTION IN ZEBRAFISH

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S.A.P. conceived this work. Z.B. designed, collected, analysed and interpreted the results from studies related to the characterization of the C9-miR zebrafish line and synaptic defects. Y.E.P. collected and analysed the results of Co-IP experiments. J.G. and S.A.P. generated the C9-miR zebrafish line. S.A.P. and Z.B. performed the electrophysiological analyses. Z.B., J.G., Y.E.P. and S.A.P. interpreted the results. S.A.P. secured the research funding. Z.B., J.G. and S.A.P. drafted the manuscript. Z.B. and S.A.P. with contributions from all authors prepared the final version of the manuscript. All authors read the final version of this manuscript.

2.1 Abstract

The most common genetic cause of amyotrophic lateral sclerosis (ALS) and fronto-temporal dementia (FTD) is a hexanucleotide repeat expansion within the C9orf72 gene. Reduced levels of C9orf72 mRNA and protein have been found in ALS/FTD patients, but the role of this protein in disease pathogenesis is still poorly understood. Here, we report the generation and characterization of a stable C9orf72 loss-of-function (LOF) model in the zebrafish. We show that reduced C9orf72 function leads to motor defects, muscle atrophy, motor neuron loss and mortality in early larval and adult stages. Analysis of the structure and function of the neuromuscular junctions (NMJs) of the larvae, reveal a marked reduction in the number of presynaptic and postsynaptic structures and an impaired release of quantal synaptic vesicles at the NMJ. Strikingly, we demonstrate a downregulation of SV2a upon C9orf72-LOF and a reduced rate of synaptic vesicle cycling. Furthermore, we show a reduced number and size of Rab3a-postive synaptic puncta at NMJs. Altogether, these results reveal a key function for C9orf72 in the control of presynaptic vesicle trafficking and release at the zebrafish larval NMJ. Our study demonstrates an important role for C9orf72 in ALS/FTD pathogenesis, where it regulates synaptic vesicle release and neuromuscular functions.

Keywords

ALS, C9orf72, synapses, motor neuron, zebrafish

2.2 Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive and ultimately lethal neuromuscular disease involving the degeneration and loss of motor neurons. Current Food and Drug Administration-approved treatments for ALS are only modestly effective and the disease still results in complete paralysis and death within the first 5 years after diagnosis. GGGGCC hexanucleotide repeat expansions within the first intron of C9orf72 is the most common genetic cause of ALS and frontotemporal dementia (FTD) (DeJesus-Hernandez *et al.*, 2011; Renton *et al.*, 2011). The pathogenic mechanism by which the repeat expansions cause disease may involve toxic gain-of function (GOF) mechanisms, such as RNA toxicity (Zu *et al.*, 2013) and protein toxicity, by aberrant dipeptide repeat protein (DPR) accumulation (Ash *et al.*, 2013; Mori *et al.*, 2013a). Alternatively, reduced C9orf72 mRNA and protein levels in a range of patient tissues and patient-derived cell lines (Belzil *et al.*, 2013; DeJesus-Hernandez *et al.*, 2011; Frick *et al.*, 2018) suggest that loss of function (LOF) by C9orf72 haploinsufficiency may also contribute to C9orf72 ALS/FTD.

The two GOF pathogenic mechanisms are extensively studied (Balendra *et al.*, 2018), while the role of C9orf72-LOF in ALS pathogenesis remains poorly understood. Importantly, in general, how the GGGGCC hexanucleotide repeat expansions cause neurodegeneration in ALS and FTD is still uncertain. The C9orf72 protein has been shown to function in a complex with the WDR41 and SMCR proteins as a guanine exchanging factor (GEF) for Rab8 and Rab39 (Corbier *et al.*, 2017; Sullivan *et al.*, 2016). It has also been proposed to play a role in autophagic flux (Sullivan *et al.*, 2016; Ugolino *et al.*, 2016; Yang *et al.*, 2016), endosomal trafficking (Sellier *et al.*, 2016; Shi *et al.*, 2018; Zhang *et al.*, 2018c) and regulating AMPA receptor levels (Xiao *et al.*, 2019).

Synaptic alterations at neuromuscular junctions (NMJs) have been found in ALS patients and in animal models of ALS. For instance, Killian et al. observed that initial compound motor action potentials (CMAP) in ALS patients were of low amplitude but did not demonstrate early post-exercise facilitation (reduction in decrement occurred at 3 min post-exercise). The low baseline CMAP amplitudes with decrement may suggest a presynaptic transmission deficit (Maselli *et al.*, 1993). In vitro microelectrode studies of ALS patient anconeus muscle demonstrated reduced presynaptic acetylcholine quantal stores, possibly explained by the diminished size of nerve terminals (Maselli *et al.*, 1993). In mutant SOD1-expressing mice (Fischer *et al.*, 2004; Frey *et al.*, 2000) an early retraction of presynaptic motor endings was observed long before the death of motoneurons (Murray *et al.*, 2010). Such an observation was also observed in tissue from patients with ALS (Maselli *et al.*, 1993). In zebrafish, expression of mutant human TARDBPG348C mRNA or FUSR521H resulted in impaired transmission, reduced frequency of miniature endplate

currents (mEPCs) and reduced quantal transmission at the NMJ (Armstrong *et al.*, 2013a; Armstrong *et al.*, 2013b). C9orf72 is expressed presynaptically and postsynaptically (Xiao *et al.*, 2019). The function of C9orf72 at synapses remains interesting and largely unexplored, yet a full understanding of its synaptic function can extend its contribution to ALS pathogenesis and uncover therapeutic targets.

Zebrafish is a powerful tool for studying neurological diseases relevant to humans, including ALS (Patten *et al.*, 2017). Using a stable transgenic zebrafish model with reduced C9orf72 expression, we analysed the effects of reduced C9orf72 function on the zebrafish's neuromuscular system. These zebrafish display behavioural deficits and early mortality as observed in C9orf72-ALS patients. C9orf72 silencing resulted in impaired synaptic activity and downregulation of the synaptic protein, synaptic vesicle (SV)- associated protein 2a (SV2a). Our findings suggest that LOF mechanisms underlie defects in synaptic function in C9-ALS.

2.3 Results

2.3.1 Generation of stable C9orf72-LOF model in zebrafish

To better understand the role of C9orf72-LOF in ALS/FTD pathogenesis, we generated a stable transgenic zebrafish gene-silencing model. A single conserved C9ORF72 orthologue is present in zebrafish on its chromosome 13. To achieve transgenic c9orf72 gene silencing in zebrafish, we used a recent miRNA-based gene-silencing approach developed for zebrafish (Giacomotto *et al.*, 2015). Unlike morpholino-based knockdown approach, transgenic zebrafish lines that have been constructed to stably express miRNAs designed to target knockdown desired genes of interest have no apparent nonspecific toxic effects (Leong *et al.*, 2012). The miRNA knockdown technique consists in the use of transgenic DNA construct allowing the expression of synthetic miRNA targeting the 3'-untranslated region (3'-UTR) of a gene of interest, here the endogenous zebrafish c9orf72 (Fig. 2.1a). As presented more in detail in the 'Methods' section, we designed 4× different miRNAs targeting specifically c9orf72 (C9orf72-miR) that we inserted downstream of a dsRED marker and under the control of a ubiquitous promoter (Ubiquitin), and the overall sequence was recombined into a mini Tol2-R4R2 destination plasmid. To generate a transgenic line, this Tol2-DNA construct was co-injected with transposase mRNA in fertilized eggs at the one-cell stage for enhanced genomic integration of the DNA construct (Kawakami *et al.*, 2000). To ease the selection of the founders/carriers, we also included an enhanced green fluorescent protein (eGFP) cassette under the crystallin promoter (Fig. 2.1a). Founders with eyes displaying GFP fluorescence (Fig. 2.1b) were selected and raised to generate a stable and heritable C9orf72-miR

LOF line (hereafter referred as C9-miR). F1 transgenic fish gave a birth to a ratio of close to 50% positive GFP embryos when outcrossed with wild-type animals, suggesting the presence of a single genomic insertion.

We first analyzed C9orf72 silencing efficiency in our C9-miR line by reverse transcriptase quantitative PCR (RT-qPCR) and western blotting. We showed a significant decrease in the level of C9orf72 mRNA (Fig. 2.1c) associated with a 50% decrease of C9orf72 protein (Fig. 2.1d, e and Supplementary Fig. 1). Altogether, these results indicate that our genetic approach efficiently reduces the C9orf72 protein levels in vivo, and this C9-miR line can be used to understand the role of C9orf72 haploinsufficiency in ALS.

2.3.2 C9orf72-LOF model shows early motor behavioural defects and reduced viability

We did not observe any overt morphological abnormalities during embryonic development (0–5 days post fertilization (dpf)) in C9-miR fish (Fig. 2.1f). From 6 to 14 dpf, some C9-miR larvae exhibited gradual morphological defects such as an unusual body curve and premature death (Fig. 2.1f, g). C9orf72 partial depletion importantly led to a significant decrease in survival at 10 dpf compared to wild-type controls, with a survival rate of 2–5% after 15 dpf (Fig. 2.1g).

We next examined whether normal zebrafish motor behaviour was affected in larval C9-miR zebrafish (4–11 dpf). To assess motor activity, larval zebrafish that did not display any of the abnormal morphological defects were selected and monitored using the automated Noldus Ethovision XT behaviour monitoring system (Fig. 2.2a). A significant decrease in motor activity was observed in C9-miR fish as compared to controls, as of 6 dpf (Fig. 2.2a, b). Such an impaired motor behaviour early on in C9-miR zebrafish is consistent with findings that we and others have reported in several other zebrafish models of ALS (Da Costa *et al.*, 2014; Patten *et al.*, 2017; Sakowski *et al.*, 2012; Shaw *et al.*, 2018). To test the specificity of the zebrafish C9orf72-LOF motor behavioural phenotype, mRNA encoding the human C9orf72 long transcript (C9-rescue) was injected into C9-miR fish, which significantly rescued the motor behavioural defects (Fig. 2.2a, b).

2.3.3 C9orf72 silencing affects NMJ structural integrity and quantal release

To understand the underlying causes of motor behavioural defects at 6 dpf in C9-miR fish, we next examined NMJ integrity by performing double immunohistochemistry at larval stages using specific presynaptic (SV2) and postsynaptic markers (α-bungarotoxin). In 6 dpf C9-miR larvae,

we observed a significant reduction in the number of colocalizing presynaptic and postsynaptic puncta (Fig. 2.2c, d) compared to control larvae. These NMJ anomalies were significantly rescued upon expression of human C9orf72 long transcript (Fig. 2.2c, d). Analysis revealed no change in the primary motor neuron axon architecture and in the colocalization of presynaptic and postsynaptic signals in C9-miR fish at early developmental stages (2 dpf; Supplementary Fig. 2). Altogether, these results indicate that, while the synaptic structures of the NMJ develop properly and are preserved at early larval stages in C9-miR, they do start to degenerate as of 6 dpf.

To investigate whether alterations in NMJ integrity had functional consequences on synaptic transmission in the 6 dpf C9-miR larvae, we recorded and analyzed the spontaneous mEPCs that occur naturally and spontaneously at synapses and represent the unitary event during synaptic transmission (Fig. 2.3a, b). We observed that the frequency of mEPCs in C9-miR was significantly reduced compared to controls (Fig. 2.3c), suggesting a reduction in the number of functional presynaptic endings. The mean amplitude of mEPCs was also found to be smaller in zebrafish C9-miR compared to wild-type zebrafish (Fig. 2.3d). We observed that the mEPCs from the muscle of C9-miR larvae and controls shared similar rise time and decay time constant kinetics (Fig. 2.3e, f).

2.3.4 C9orf72-LOF model displays TDP-43 pathology

Cytoplasmic aggregation of trans-activation response element (TAR) DNA-binding protein 43 (TDP-43) is a major pathological hallmark of ALS (Neumann *et al.*, 2006). TDP-43 forms aggregates in neurons, glial cells (Neumann *et al.*, 2006) and axial skeletal muscles (Mori *et al.*, 2019). By taking advantage of the relatively large nucleus and cytoplasm of the skeletal muscle cells (Fig. 2.4a), we examined whether TDP-43 pathology exist in our model. Using a specific antibody that recognizes the highly homologous human TDP-43 orthologue in zebrafish (Schmid *et al.*, 2013b), we showed that this protein is localized to the nucleus of the skeletal muscle cells at 6 dpf in wild-type zebrafish (Fig. 2.4a). In contrast, in C9-miR zebrafish, we observed clusters of TDP-43 that are predominantly located outside of the nucleus of muscle cells (Fig. 2.4b and Supplementary Fig. 2b). Quantitative analysis revealed a significant reduction of the nuclear-to cytoplasmic (N-to-C) ratio of TDP-43 in C9-miR fish compared to controls, evidencing the cytoplasmic accumulation of TDP-43 in the muscles of C9-miR fish (Fig. 2.4c). This TDP-43 pathology in C9-miR zebrafish was significantly rescued upon expression of the human C9orf72 mRNA (Fig. 2.4b, c). Altogether, our findings provide strong evidence that C9orf72 silencing in zebrafish recapitulates a key pathological hallmark of ALS.

2.3.5 C9orf72-LOF zebrafish model display adult hallmark features of ALS

C9-miR fish that survive past 15 dpf were also studied at adult stages for hallmarks of ALS such as motor behavioural anomalies/paralysis and neuromuscular deficits. At the motor behavioural level in 12 months fish, we observed an impaired swimming ability in adult C9-miR animal compared to controls (Fig. 2.5a, b and Supplementary Movies 1 and 2). Prior to death, C9-miR fish spent their time in the bottom of the tank with weak movements and showing signs of paralysis (a stage that we termed 'end stage'). Adult survival was also monitored and we observed that by 16 months post-fertilization, >80–90% of the adult C9-miR zebrafish die. We next investigated neuromuscular pathology by first examining NMJ integrity in 12-month-old control and C9-miR fish (Fig. 2.5c). Quantification of the number of colocalized presynaptic (SV2) and postsynaptic (α-bungarotoxin) puncta revealed a significantly reduced number of synaptic puncta at NMJs in adult C9-miR fish compared to adult control fish (Fig. 2.5d). NMJ degeneration is followed by motoneuron loss in ALS patients and animal models (Fischer *et al.*, 2004; Frey *et al.*, 2000). Using choline acetyltransferase (ChAT) staining, we next examined the motor neurons in the spinal cord of C9-miR and control adult fish. Motor neurons in the zebrafish spinal cord are small or large depending on the maturation stage of the neurons and vary in physiology (McLean *et al.*, 2007; Menelaou *et al.*, 2012). Large-sized motor neurons (≥10 μm in diameter) are fast-fatigable motor neurons that are most vulnerable to degeneration (Roselli *et al.*, 2014). We observed an overall reduction in the total number of ChAT-positive motor neurons in C9-miR fish (Fig. 2.5e) and the mature motor neurons (i.e. large cell body) were reduced in size compared to control fish (Fig. 2.5e, f), consistent with motor neuron degeneration pathology in ALS patients. Hematoxylin and eosin (H&E) staining of cross-section of fish body trunk revealed that the muscles in adult C9-miR exhibited severe atrophy (Fig. 2.5g), with a significant reduction in the thickness of the fibres (Fig. 2.5g, h).

Given that the nuclei of motor neurons are large in size and easily examined in adult spinal cord sections, we examined whether TDP-43 pathology occur in spinal motor neurons in C9-miR animals. Cells in the spinal sections of 14- to 16-month-old fish were stained for ChAT, TDP-43 and 4,6-diamidino-2-phenylindole (DAPI) (Fig. 2.6). Strikingly, we observed unusual circular clustering of TDP-43 stains that are negative for both DAPI staining in the grey matter of the spinal cord (Fig. 2.6a, b), indicating that TDP-43 clusters reside in the cytoplasm. Since the expression of TDP-43 is dispersed in wild-type control fish (Fig. 2.6a) and that neurons in the zebrafish spinal cord are in very close proximity, this complicates the quantification of the N-to-C expression level of TDP-43. Given that nuclear depletion of TDP-43 has been reported as a measure of TDP-43

pathology in several studies (Geser *et al.*, 2008; Velebit *et al.*, 2020; Winton *et al.*, 2008a), we thus quantified the percentage colocalization between the cellular TDP-43 antibody signal and the nuclear DAPI stain in ChAT+ motor neurons in control and C9-miR spinal cord sections. Compared to controls, the degree of colocalization of TDP-43 signal and DAPI stain in spinal motor neurons was significantly reduced, suggesting a nuclear depletion in C9-miR motor neurons (Fig. 2.6a–c).

2.3.6 C9orf72 regulates synaptic vesicle exocytosis and synapse stability at the NMJ

To gain molecular insights into the function of C9orf72, we examined the processes and pathways affected in C9-miR fish by determining global changes at the proteomic levels by isolating total proteins at 6 dpf from C9-miR larvae and wild-type siblings. We identified a total of 2602 proteins, out of which 2093 proteins were covered by ≥2 unique peptides and were quantifiable in 4 biological replicates (false discovery rate ≤1%; Fig. 2.7a). Most of the proteins in wild-type and C9-miR fish were at comparable expression levels. Only 24 proteins were found to be dysregulated ($p < 0.05$; log2 fold-change of −1.5 and 1.5; Supplementary Table 2). Of these hits, 12 were upregulated and 12 were downregulated in C9-miR fish (Fig. 2.7b). These differentially expressed proteins (DEPs) were classified into functional clusters according to the PANTHER classification system (Supplementary Fig. 3). The classification results revealed that many DEPs were distributed into six protein classes (Supplementary Fig. 3a). These proteins are classified into three molecular functions, namely binding (20%), structural molecule activity (20%) and catalytic activity (60%) (Supplementary Fig. 3b). They are involved in biological processes, with cellular process, metabolic process and biological regulations being the most represented ones with 38%, 23.1% and 15.4% of proteins, respectively (Supplementary Fig. 3c). Cellular component analysis revealed that the DEPs belong in majority to the organelle, membrane and synapse categories (Supplementary Fig. 3d). Consistent with the synaptic dysfunction phenotype, we identified a strong downregulation of synaptic proteins (Fig. 2.7b and Supplementary Table 2). Among these proteins, the top hit of dysregulated proteins is the synaptic protein SV2a. Importantly, a recent study showed that SV2a is reduced in C9orf72-ALS patient-derived induced pluripotent stem cell (iPSC) neurons (Jensen *et al.*, 2020). This data links the findings in our C9orf72-LOF model to ALS. In order to test whether C9orf72 interacts with SV2a, we performed coimmunoprecipitation coupled with western blot. HEK293T cells were co-transfected with RFP-C9orf72 with Myc-SV2a or Myc-SMCR8, a known interaction partner of C9orf72 as positive

control (Sellier *et al.*, 2016). SMCR8 and SV2a were also co-transfected with red fluorescent protein (RFP) as negative control. We found that Myc-SV2a was co-immunoprecipitated by RFP-C9orf72 but not by the RFP control (Fig. 2.7c). Taken together, these results suggest that C9orf72 interacts with SV2a. Given that SV2a is an essential component of active zones and synaptic release machinery, we next sought to further assess synaptic activity at the NMJ by measuring SV cycling at the NMJ in zebrafish larvae using the fluorescent styryl dye, FM1-43 (Li *et al.*, 2003; Wei *et al.*, 2013). C9-miR and control larvae were exposed to FM1-43 and its uptake into NMJ presynaptic boutons was monitored. The presynaptic terminals were acutely depolarized with a high [K+] Hank's Balanced Salt Solution (HBSS) (45mM) to drive the exocytotic activity, SV cycle, load FM1-43 and label synaptic clusters. In controls, we observed strong fluorescence staining along terminal axon branches at individual synaptic varicosity boutons (Fig. 2.8a), while in C9-miR fish we found a significant reduction in FM1-43 loading in presynaptic terminals (Fig. 2.8b), indicating slowing of the exocytotic activity and the overall SV cycle. These findings reveal a key role for C9orf72 in regulating pre-SV release at NMJ. To assess the organization of the presynaptic structure at the NMJ, we examined the expression of Rab3a, a protein associated with vesicles at active zones that is essential for SV release and neurotransmission (Fig. 2.8c–e). We found a reduced number of Rab3+ puncta in C9-miR fish compared to controls (Fig. 2.8c, d). Additionally, the area of the putative synapses were smaller in C9-miR fish (Fig. 2.8e) compared to control fish.

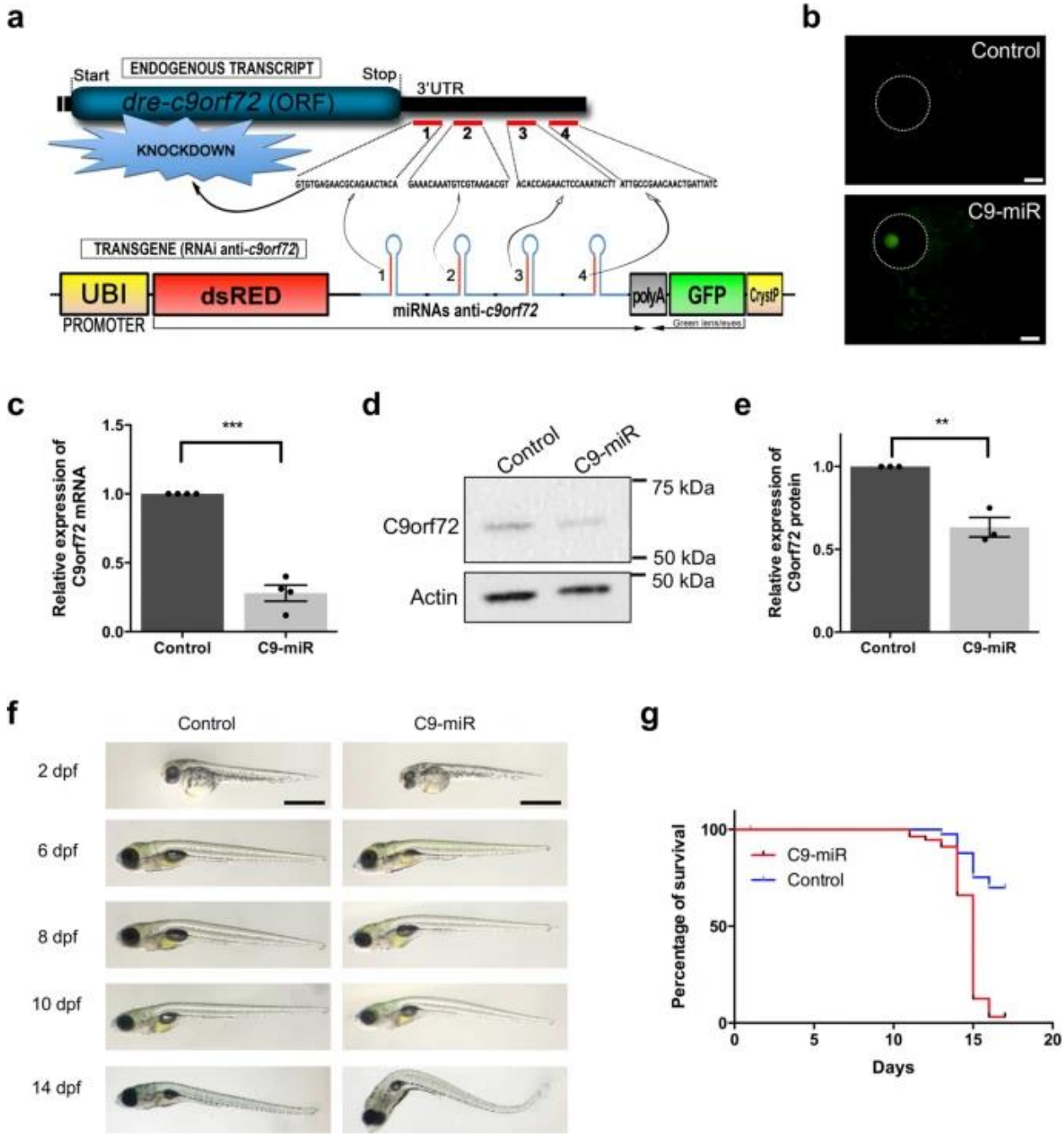

a
Start
ENDOGENOUS TRANSCRIPT
Stop
3'UTR
dre-c9orf72 (ORF)
KNOCKDOWN
1
2
3
4
GTGTGAGAACGCAGAACTACA
GAAACAAATGTCGTAAGACGT
ACACCAGAACTCCAAATACTT
ATTGCCGAACAACTGATTATC
TRANSGENE (RNAi anti-c9orf72)
UBI
PROMOTER
dsRED
miRNAs anti-c9orf72
polyA
GFP
CrystP
Green lens/eyes
b
Control
C9-miR
c
Relative expression of C9orf72 mRNA
***
1.5
1.0
0.5
0.0
Control
C9-miR
d
Control
C9-miR
C9orf72
Actin
75 kDa
50 kDa
50 kDa
e
Relative expression of C9orf72 protein
**
1.0
0.5
0.0
Control
C9-miR
f
Control
C9-miR
2 dpf
6 dpf
8 dpf
10 dpf
14 dpf
g
Percentage of survival
100
50
0
C9-miR
Control
0
5
10
15
20
Days

Figure 2-1: Generation of a stable zebrafish C9orf72 knockdown line.

a Schematic representation of the technique used to silence *C9orf72* in zebrafish. The transgene is designed to express four different micro-RNAs targeting C9orf72's 3'UTR and triggering knockdown by both repressing *C9orf72* translation and affecting its stability. **b** Images demonstrating proper eGFP expression in the crystallin of the transgenic fish, a marker used to identify carrier/knockdown larvae. Scale bar = 100 µm. **c** Bar graph shows the relative expression of the endogenous *C9orf72* gene. mRNA was normalized to elf1α mRNA levels ($N = 4$, $^{***}p < 0.0001$, Student's *t* test). **d** Immunoblotting of the zebrafish protein C9orf72 and beta-actin as control. **e** Bar graph shows the relative expression of the C9orf72 protein compared with actin between C9orf72 mutants and control line ($N = 3$; $^{**}p = 0.0034$; Student's *t* test). **f** Gross morphological analyses of wild-type control and C9orf72-LOF fish (C9-miR). **g** Kaplan–Meier survival plot over 17 days after fertilization (dpf) showing low survival of C9-miR compared to controls after 10 dpf ($N = 3$, $n = 25$). Data are presented as mean ± SEM. *n* represents the number of fish, *N* represents the number of experimental repeats.

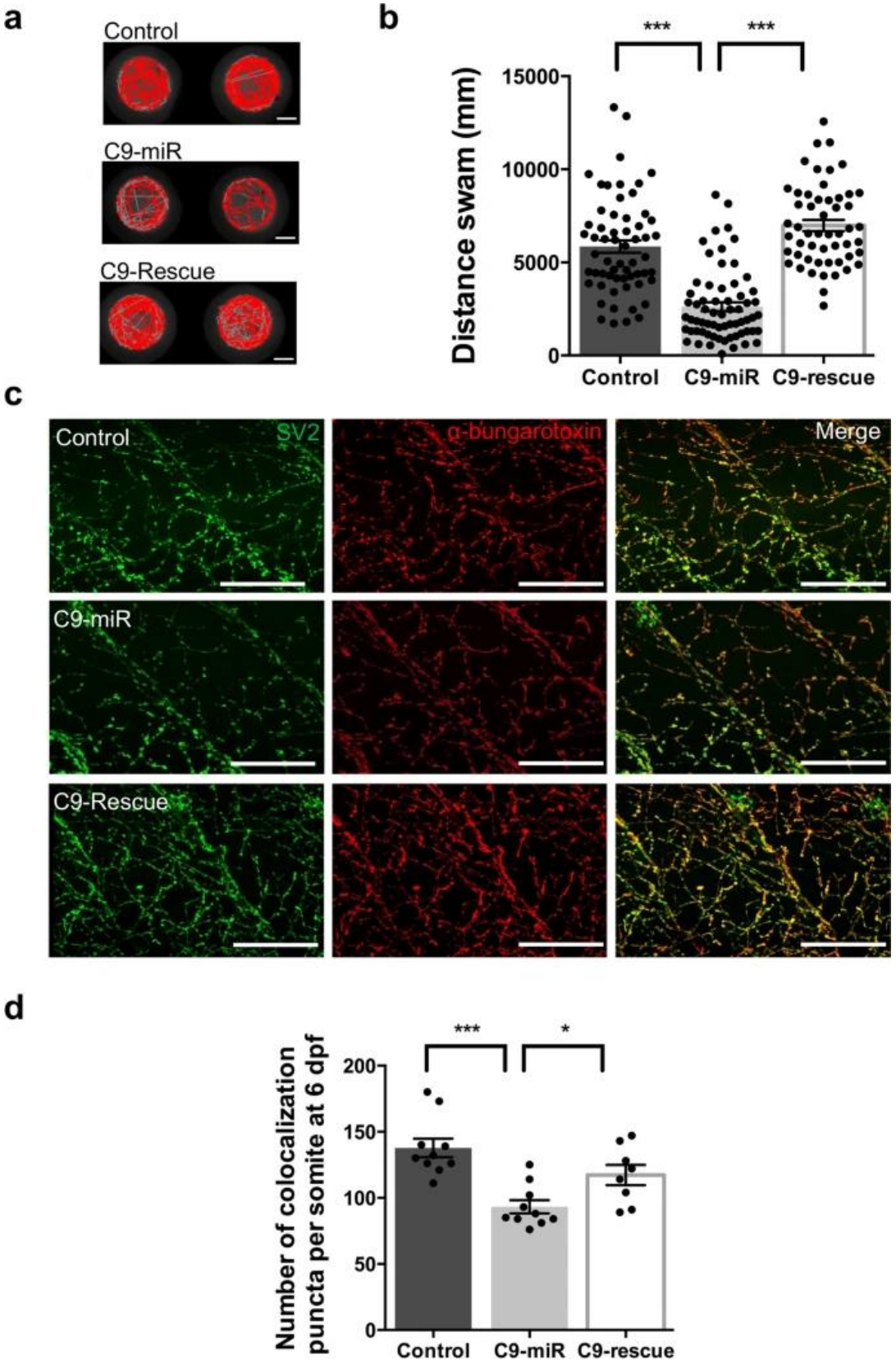
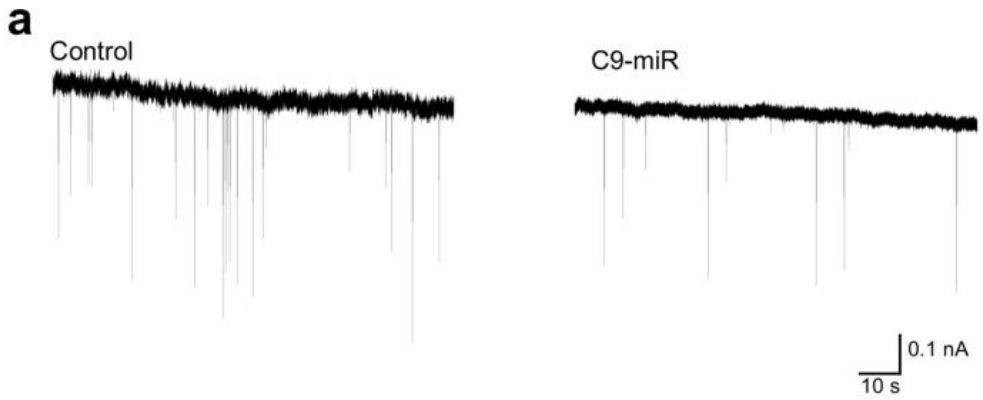
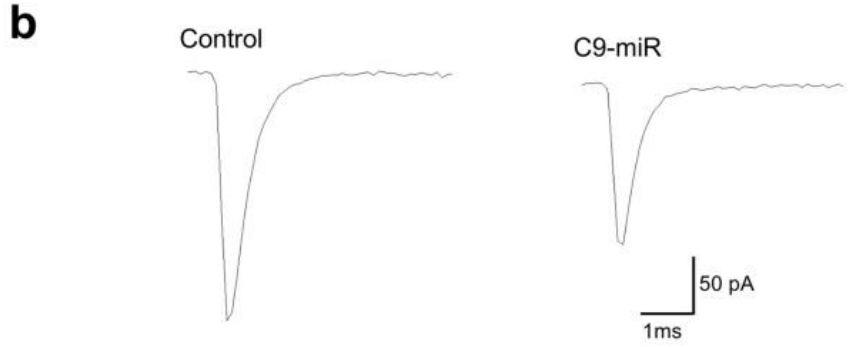
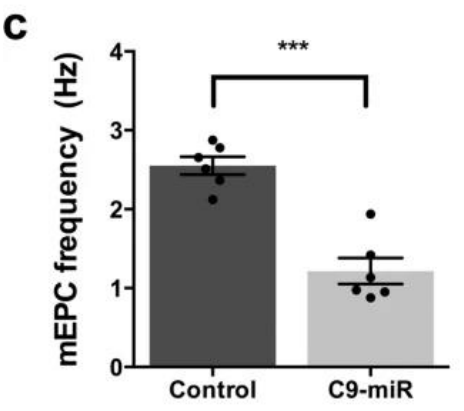

a
Control
C9-miR
C9-Rescue
b
***
***
Distance swam (mm)
15000
10000
5000
0
Control
C9-miR
C9-rescue
c
Control
SV2
α-bungarotoxin
Merge
C9-miR
C9-Rescue
d
***
*
Number of colocalization puncta per somite at 6 dpf
200
150
100
50
0
Control
C9-miR
C9-rescue

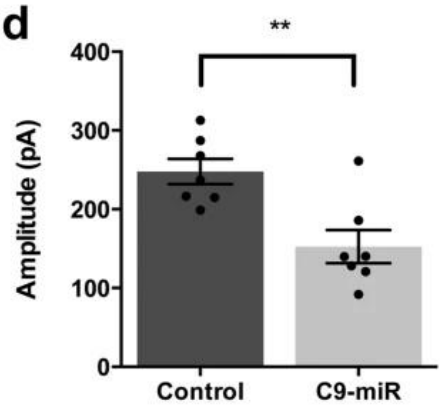
Figure 2-2: Motor behavioural deficits and reduced acetylcholine receptor clusters at neuromuscular junctions (NMJs) in C9-miR fish.

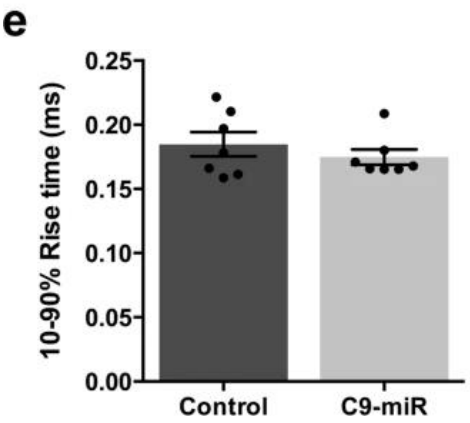
a Representative swimming tracks of control, C9-miR and C9-rescue fish at 6 dpf. Scale bar = 0.2 cm. **b** C9-miR larvae ($N = 3$, $n = 65$) displayed impaired swimming compared to controls ($N = 3$, $n = 60$; ***$p < 0.0001$; one-way ANOVA). Expression of the human *C9orf72* long transcript in C9-miR fish (C9-rescue; $N = 3$, $n = 53$) significantly rescued the motor behavioural defects (***$p < 0.0001$; one-way ANOVA). **c** Representative images of co-immunostaining of zebrafish neuromuscular junctions with presynaptic (SV2; green) and postsynaptic (α-bungarotoxin; red) markers in 6 dpf zebrafish. Scale bar = 100 µm. **d** Quantification of the colocalizing presynaptic and postsynaptic markers per somite showed a significant reduction in the number of puncta in C9-miR fish at 6 dpf that can be rescued with the expression of human *C9orf72* mRNA (C9-rescue) ($n = 8–10$; ***$p < 0.0001$; one-way ANOVA). Data are presented as mean ± SEM. *n* represents the number of fish, *N* represents the number of experimental repeats.


a
Control
C9-miR
0.1 nA
10 s


b
Control
C9-miR
50 pA
1ms


c
mEPC frequency (Hz)
***
Control
C9-miR


d
Amplitude (pA)
**
Control
C9-miR


e
10-90% Rise time (ms)
Control
C9-miR

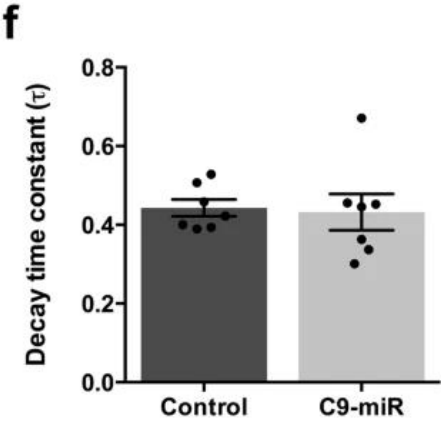

f
Decay time constant (τ)
Control
C9-miR

Figure 2-3: C9-miR zebrafish exhibited attenuated miniature endplate currents (mEPCs) at NMJs.

a Recordings of mEPCs, which result from spontaneous release of a quantum, were recorded in 6 dpf control and C9-miR fish ($n = 6$). **b** Representative mEPCs. Animals with reduced C9orf72 (C9-miR) displayed mEPCs with reduced frequency (**c**) ($n = 6–7$; ***$p < 0.0001$; Student's *t* test) and amplitude (**d**) ($n = 7$; **$p < 0.001$; Student's *t* test). Rise time (**e**) ($n = 7$; $p = 0.3947$; Student's *t* test) and decay time (**f**) constant kinetics of mEPC ($n = 7$; $p = 0.8385$; Student's *t* test) were not found to be significantly different between controls and C9-miR. Data are presented as mean ± SEM. *n* represents the number of fish.

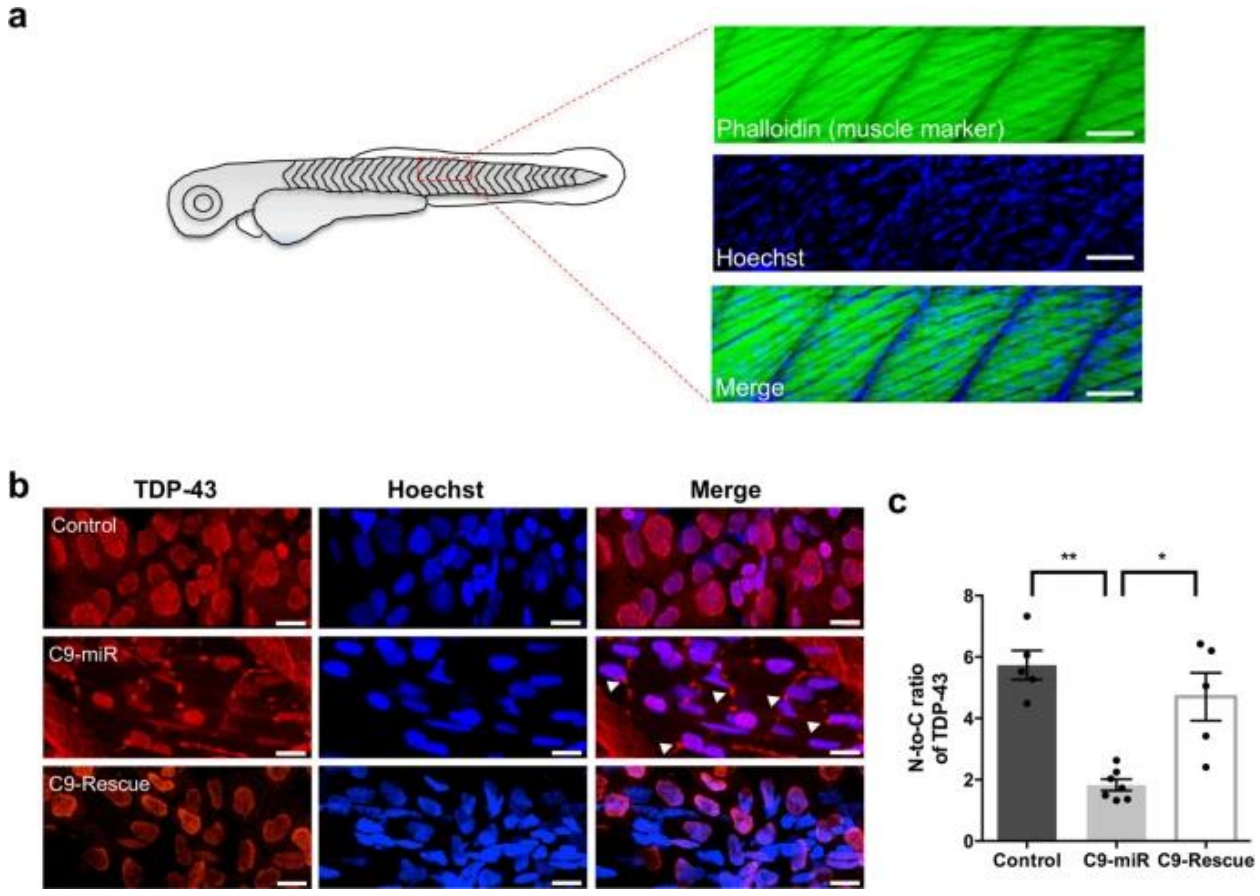

a
Phalloidin (muscle marker)
Hoechst
Merge
b
TDP-43
Hoechst
Merge
Control
C9-miR
C9-Rescue
c
N-to-C ratio of TDP-43
**
*
0
2
4
6
8
Control
C9-miR
C9-Rescue

Figure 2-4: C9-miR zebrafish displayed TDP-43 pathology.

a Illustration of 6 dpf zebrafish skeletal muscle cells with large nuclei labelled with muscle marker, phalloidin, and nucleus marker, Hoechst. **b** Representative images of 6 dpf zebrafish skeletal muscle cells for TDP-43. Compared to control fish, we observed cytoplasmic clustering of TDP-43 expression in the C9-miR skeletal muscles that can be rescued in C9-rescue fish. Arrows indicate clusters of TDP-43 expression. **c** Quantification of the nucleus-to-cytoplasmic ratio for TDP-43. A significant reduction in N-to-C for TDP-43 was observed in C9-miR zebrafish ($n = 7$; $^{**}p < 0.005$; one-way ANOVA) that can be significantly rescued upon the expression of the human *C9orf72* mRNA ($n = 5$; $^{*}p < 0.05$; one-way ANOVA). Scale bar = 50 µm. Data are presented as mean ± SEM. *n* represents the number of fish.

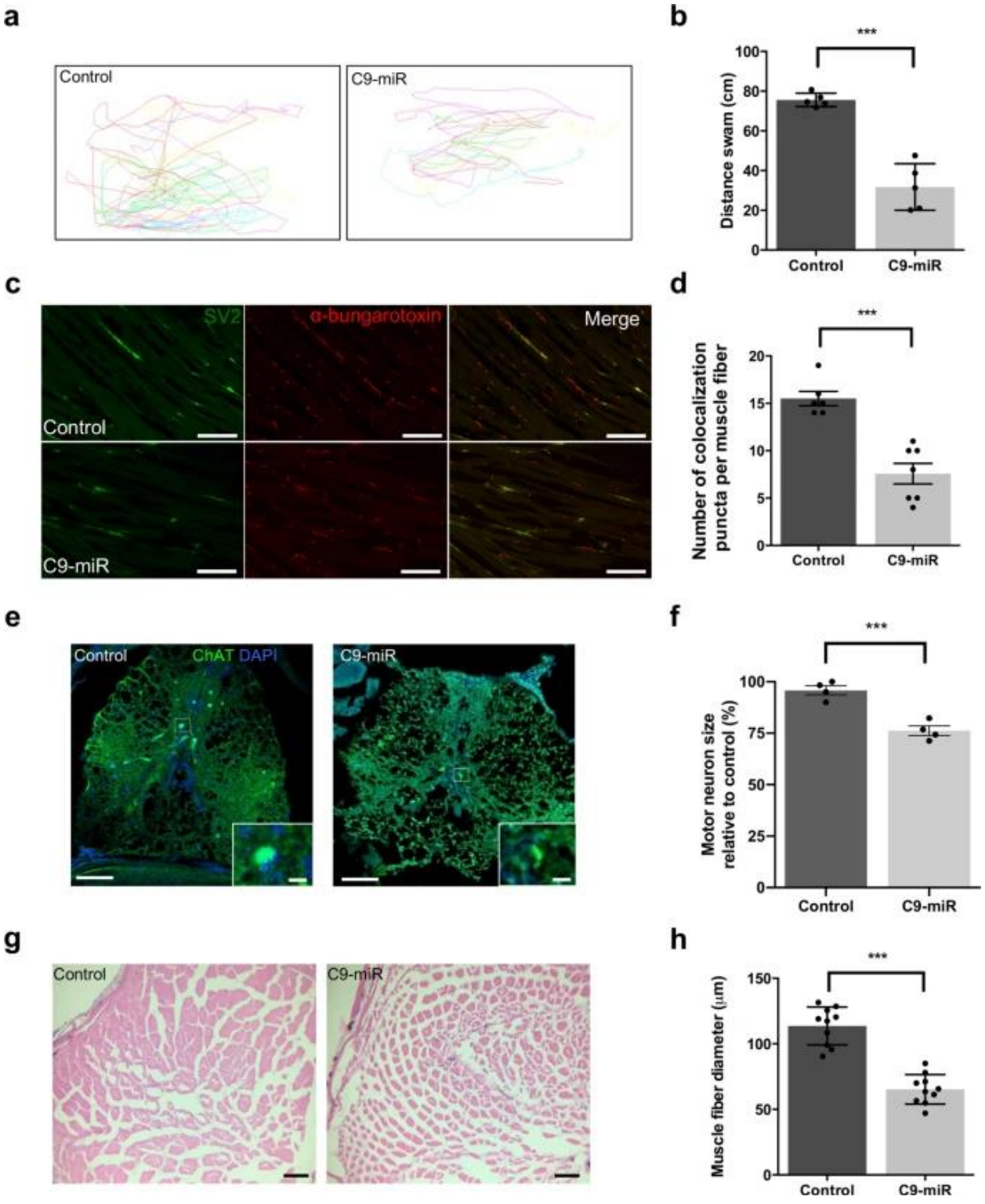

a
Control
C9-miR
b
***
Distance swam (cm)
100
80
60
40
20
0
Control
C9-miR
c
SV2
α-bungarotoxin
Merge
Control
C9-miR
d
***
Number of colocalization puncta per muscle fiber
20
15
10
5
0
Control
C9-miR
e
Control
ChAT DAPI
C9-miR
f
***
Motor neuron size relative to control (%)
100
75
50
25
0
Control
C9-miR
g
Control
C9-miR
h
***
Muscle fiber diameter (μm)
150
100
50
0
Control
C9-miR

Figure 2-5: Adult zebrafish C9-miR display muscle atrophy, smaller motoneurons and behavioural deficits.

a Representative traces of swimming activity of five adult controls and C9-miR fishes (12-month old) during 30 s (left panel). **b** C9-miR fish exhibit behavioural deficits (right panel) ($n = 5$, ***$p < 0.0001$, Student's *t* test). **c** Adult 12-month-old NMJs were examined in trunk section by co-immunostaining SV2 (green) and α-bungarotoxin (red). **d** Quantification of the colocalizing presynaptic and postsynaptic clusters at NMJ in adult (12-month old) wild-type control ($n = 6$) and C9-miR fish ($n = 7$) (***$p < 0.0001$, Student's *t* test). **e** ChAT staining in adult zebrafish spinal cord. **f** Large (mature) motor neurons (inset in e; scale bar = 10 µm) are reduced in size in C9-miR compared to controls ($n = 4$; ***$p < 0.0001$, Student's *t* test). **g** Examination of adult zebrafish muscle myotomes by haematoxylin and eosin staining. **h** C9-miR fish display a smaller diameter of muscle fibres compared to controls ($n = 10$; ***$p < 0.0001$, Student's *t* test). Data are presented as mean ± SEM. Scale bar = 50 µm. *n* represents the number of sections from three adult fish per genotype.

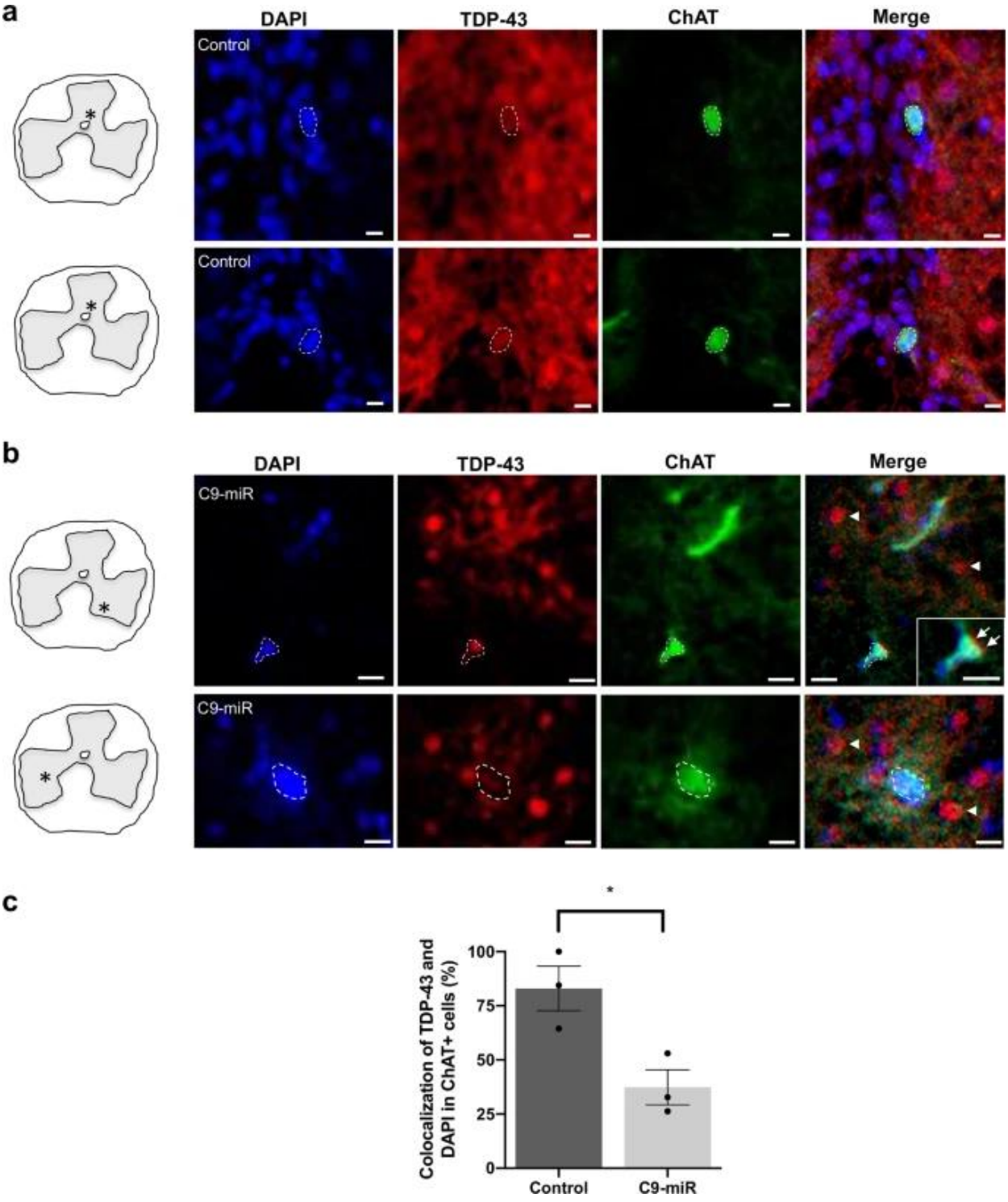
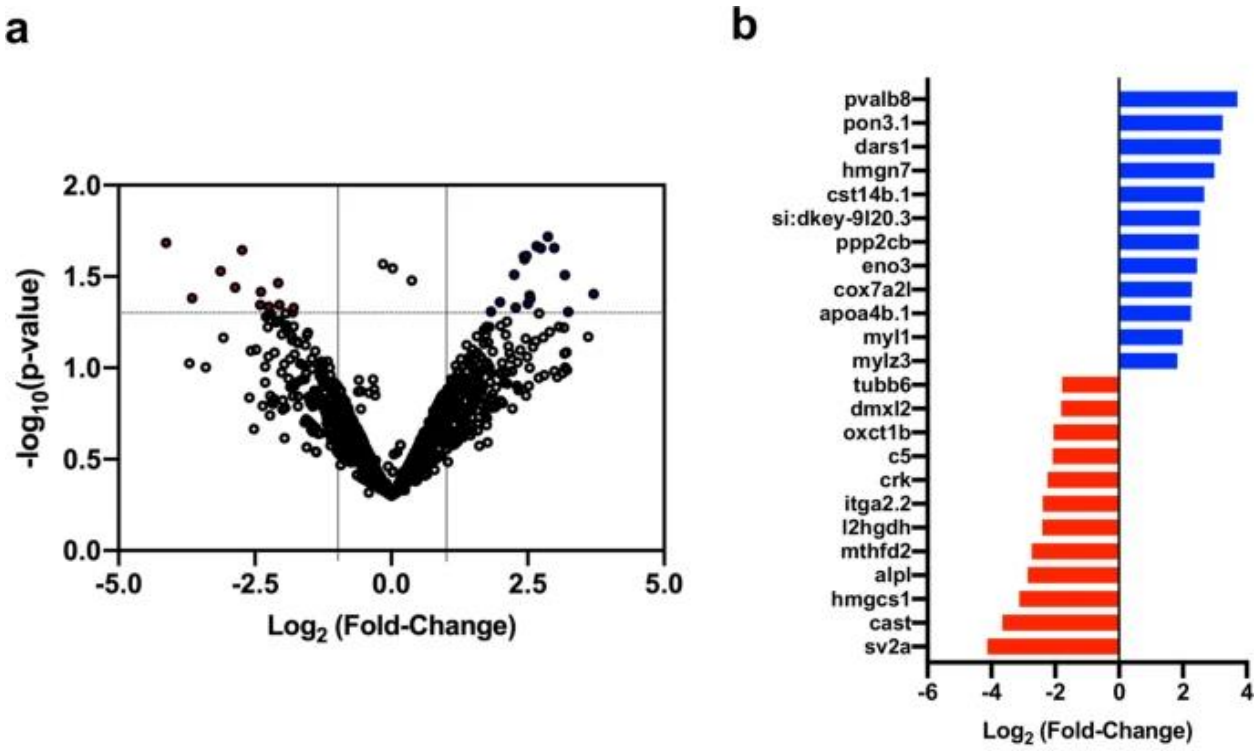

a
DAPI
TDP-43
ChAT
Merge
Control
Control
b
DAPI
TDP-43
ChAT
Merge
C9-miR
C9-miR
c
Colocalization of TDP-43 and DAPI in ChAT+ cells (%)
100
75
50
25
0
Control
C9-miR
*

Figure 2-6: TDP-43 pathology in adult C9-miR zebrafish motor neurons.

Representative fluorescence images of spinal motor neurons immunostained with antibodies against ChAT (green), TDP-43 (red) and labelled with DAPI (blue) in 14–16-month-old adult (**a**) control (wild type) or (**b**) C9-miR spinal cord sections. Scale bar = 20 µm. Arrows and arrowheads illustrate TDP-43 mislocalization. **c** Quantification of colocalization (%) between the total cellular TDP-43 antibody signal and the nuclear DAPI stain in ChAT-positive motor neurons in C9-miR fish normalized to colocalization percentage in control fish ($n = 3$; $^*p = 0.0251$, Student's t test). Data are presented as mean ± SEM. n represents the number of fish.


a
2.0
1.5
1.0
0.5
0.0
-log10(p-value)
-5.0
-2.5
0.0
2.5
5.0
Log2 (Fold-Change)
b
pvalb8
pon3.1
dars1
hmgn7
cst14b.1
si:dkey-9l20.3
ppp2cb
eno3
cox7a2l
apoa4b.1
myl1
mylz3
tubb6
dmxl2
oxct1b
c5
crk
itga2.2
l2hgdh
mthfd2
alpl
hmgcs1
cast
sv2a
-6
-4
-2
0
2
4
Log2 (Fold-Change)

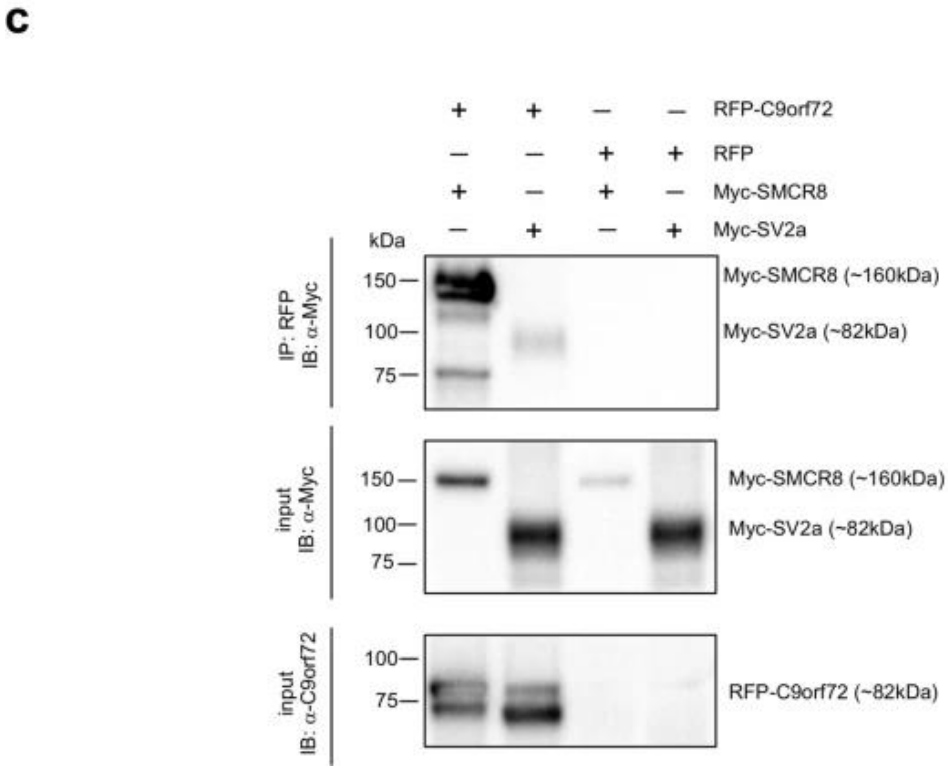

c
+ + – – RFP-C9orf72
– – + + RFP
+ – + – Myc-SMCR8
– + – + Myc-SV2a
kDa
IP: RFP
IB: α-Myc
150
100
75
Myc-SMCR8 (~160kDa)
Myc-SV2a (~82kDa)
input
IB: α-Myc
150
100
75
Myc-SMCR8 (~160kDa)
Myc-SV2a (~82kDa)
input
IB: α-C9orf72
100
75
RFP-C9orf72 (~82kDa)

Figure 2-7: Sv2a interacts with C9orf72 and its protein expression is downregulated in C9-miR fish.

a Volcano plot showing the log2 fold change against the −log10 *p* value. Proteins in blue are upregulated while proteins in red are downregulated (log2 fold change of −1.5 and 1.5, delineated by vertical lines) and are significantly dysregulated (−log10 (*p* value) > 1.301, delineated by horizontal dotted line) between control and C9-miR fish ($N = 4$). **b** Differentially expressed proteins with $p < 0.05$ in C9-miR 6 dpf larvae ($N = 4$). **c** Immunoblot analysis of RFP-immunoprecipitated proteins and lysate of HEK293T cells co-expressing RFP-tagged C9orf72 or RFP in combination with Myc-tagged SV2a or Myc-tagged SMRC8 (a known interactor of C9orf72; positive control). Myc-SV2a and Myc-SMCR8 were extracted out of solution in samples where RFP-C9orf72 was co-transfected, but not in samples where RFP was co-transfected ($N = 3$).

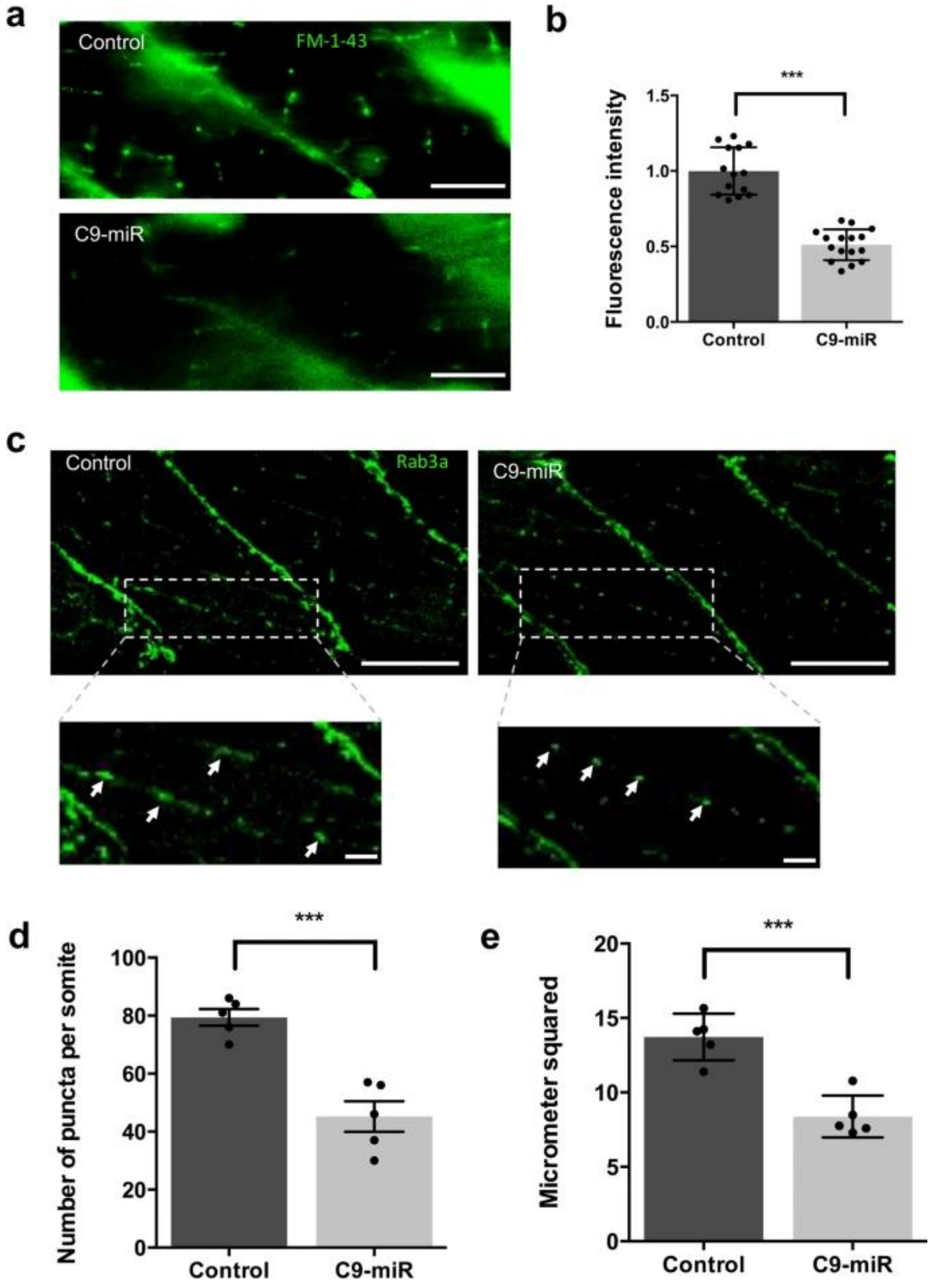

a
Control
FM-1-43
C9-miR
b
Fluorescence intensity
1.5
1.0
0.5
0.0
***
Control
C9-miR
c
Control
Rab3a
C9-miR
d
Number of puncta per somite
100
80
60
40
20
0
***
Control
C9-miR
e
Micrometer squared
20
15
10
5
0
***
Control
C9-miR

Figure 2-8: C9orf72 regulates synaptic active zones and activity at the neuromuscular junction.

a FM1-43 loading of NMJ boutons in 6 dpf fish. C9-miR fish displayed decreased FM1-43 loading compared to controls. **b** Quantification of FM1-43 fluorescent intensity in control ($n = 14$) and C9-miR fish ($n = 16$), showing a reduced FM1-43 fluorescent intensity in C9-miR fish ($p < 0.0001$; Student's t test). **c** Putative synapses (arrows) were visualized with Rab3a immunostaining at 6 dpf (inset; scale bar = 10 µm). Rab3a+ synaptic puncta were reduced in number (**d**) and area (**e**) in 6 dpf C9-miR larvae when compared with wild-type controls ($n = 5$; $^{***}p < 0.0001$; Student's t test). Scale bar = 50 µm. Data are presented as mean ± SEM. n represents the number of fish.

2.4 Discussion

Despite advances in studies of C9orf72-ALS, understanding the function of C9orf72 remains a key research element that is poorly explored. We generated a C9orf72-related ALS stable zebrafish line with a reduced expression of C9orf72. These fish display motor defects, muscle atrophy, motor neuron loss and mortality in early larval and adult stages. Additionally, they exhibit TDP-43 pathology, which is a key hallmark of ALS. Analysis of the structure and function of the NMJs revealed a significant reduction in the number of presynaptic and postsynaptic structures and an impaired release of quantal SVs at the NMJ in the C9-miR line. We also identified an important role of C9orf72 in controlling pre-SV trafficking and release at the zebrafish larval NMJ.

Reduced C9orf72 mRNA and protein levels have been reported in a range of patient tissues and patient-derived cell lines (Belzil *et al.*, 2013; DeJesus-Hernandez *et al.*, 2011; Frick *et al.*, 2018). Our C9orf72 zebrafish model provides support to a LOF mechanism underlying C9orf72-dependent ALS. Our data are consistent with deletion or transient knockdown models in Caenorhabditis elegans (Therrien *et al.*, 2013) and zebrafish (Ciura *et al.*, 2013), respectively, showing defective motor phenotypes. However, in contrast, no motor neurons deficits were reported in C9orf72 knockout mice (Atanasio *et al.*, 2016; Koppers *et al.*, 2015; Sudria-Lopez *et al.*, 2016). The phenotypic discrepancy between C9orf72 knockout mice and C9-miR fish might be explained by genetic compensation mechanisms that may occur upon complete C9orf72 deficiency (i.e. knockout) but not upon partial loss of C9orf72 (i.e. knockdown). Importantly, complete deletion of C9orf72 does not occur in C9orf72 ALS/FTD patients. In a recent elegant work, Shao and colleagues showed that C9orf72 protein dose reduction is critical for motor deficits in C9orf72 ALS/FTD mouse models (Shao *et al.*, 2019), demonstrating the importance of C9orf72 haploinsufficiency in disease manifestation in mice rather than complete loss of C9orf72. Noteworthy, although differences exist in the homology of the human C9orf72 orthologues in zebrafish and mice (i.e. 75% in zebrafish and 98% in mice), sequence differences likely have virtually little or no bearing on the phenotypic discrepancy between a higher-order organism, C9orf72 knockout mice and C9-miR fish, given that the motor deficits in C9-miR fish can be rescued by the expression of human C9orf72 mRNA. Interestingly, in human motor neurons derived from normal individual iPSCs harbouring a CRISPR/Cas9-mediated C9orf72 deletion (Shi *et al.*, 2018) and in C9orf72 knockout rats (Dong *et al.*, 2020), rapid neurodegeneration and progressive motor deficits were, respectively, noted in response to excitotoxicity.

In addition, C9orf72 knockout mice do not exhibit TDP-43 proteinopathy. The model presented here, importantly, displays TDP-43 pathology and replicates haploinsufficiency as a major

contributor to C9orf72 ALS rather than being a full ablation of C9or72 LOF model. Intriguingly, the motor phenotypes observed in C9-miR zebrafish are consistent with several other zebrafish ALS models, including zebrafish model expressing C9orf72-related repeat expansions or DPR (Shaw *et al.*, 2018; Swaminathan *et al.*, 2018; Swinnen *et al.*, 2018). However, the presence of a reduced level of C9orf72 mRNA or protein in these models, as in ALS/FTD, was not examined in these studies. Of note, the expression of GGGGCC repeat expansions or DPR in zebrafish are toxic (Lee *et al.*, 2013; Ohki *et al.*, 2017; Swaminathan *et al.*, 2018), consistent with several studies in neurons and other animals. We found that the expression of GGGGCC repeat expansions in our C9-miR fish exacerbated toxicity and resulted in death of zebrafish by 6 dpf (Supplementary Fig. 4). Such a synergistic interplay between reduced C9orf72 function and repeat-dependent gain of toxicity was observed in a recent study by Zhu and colleagues (Zhu *et al.*, 2020).

An important finding of this study is the synaptic impairments in C9-miR fish. The reduced frequencies and amplitudes of quantal neurotransmission events are consistent with observations made in several non-C9orf72 ALS models (Armstrong *et al.*, 2013a; Armstrong *et al.*, 2013b) and in tissue from patients with ALS (Maselli *et al.*, 1993).We also report significant reductions in SV exocytosis and the number and area of putative synaptic puncta at NMJs. Additionally, we show a decrease in the expression of SV protein SV2a. These findings provide a novel role of C9orf72 in synaptic physiology at the presynaptic level. Interestingly, consistent with our findings, SV2a was also recently found at reduced levels in C9orf72-ALS patient-derived iPSC neurons (Jensen *et al.*, 2020). Ablation of SV2a function in knockout models resulted in reduced number of readily releasable pool of SVs, diminished release probability and reduction in spontaneous synaptic events (Crowder *et al.*, 1999; Custer *et al.*, 2006). Intriguingly, similar observations of loss of SV2a and synaptic dysfunction were also observed in neurons expressing the C9orf72-related glycine-alanine (GA) DPR (Jensen *et al.*, 2020). DPR proteins can disrupt pre-mRNA splicing in ALS/FTD patients (Yin *et al.*, 2017). It is possible that the expression of GA DPR in neurons reduces the level of C9orf72 transcripts leading to the synaptic phenotypes. Additionally, we show that C9orf72 interacts with SV2a, suggesting that it may play a role in stabilizing the expression level of SV2a in presynaptic compartments.

Rab3a is important for transport of SVs and their docking at active zones (Leenders *et al.*, 2001). It regulates synaptic transmission and it is associated with SVs through GEF activity (Binotti *et al.*, 2016; Mahoney *et al.*, 2006). For instance, at Rab3adeficient terminals in mice, synaptic secretion response recovered slowly and incompletely following exhaustive stimulation (Leenders *et al.*, 2001). In addition, the replenishment of docked vesicles following exhaustive stimulation at

these terminals was also impaired (Leenders *et al.*, 2001). Differentially expressed in normal and neoplastic domain containing proteins such as C9orf72 can function as Rab GEFs, enabling their activation, recruitment and interaction with downstream effectors (Allaire *et al.*, 2010; Ioannou *et al.*, 2015). A previous study had identified Rab3a as part of complex interacting with C9orf72 (Frick *et al.*, 2018). Xiao et al. showed that Rab3a protein levels were unchanged in C9-WT and C9-knockout mice forebrain by western blot. Similarly, no significant change in Rab3a levels was observed in our proteomic analyses between controls and C9-miR fish. However, we observed reduced Rab3a-positive puncta at active zones at NMJs in C9-miR fish by immunostaining, suggesting that Rab3a-dependent SV transport to active zones may be impaired upon loss of C9orf72. It is, thus, plausible that in addition to the effect of reduced SV2a on synaptic dysfunction, SV exocytosis and quantal transmission defects in C9-miR fish may be exacerbated due to the altered function of C9orf72 as a GEF for Rab3a and, subsequently, its recruitment to SVs and role in SV transport.

In conclusion, we generated a stable C9orf72-LOF model in zebrafish that recapitulated some major hallmarks of ALS and enhanced our understanding of ALS pathogenesis. Importantly, our findings demonstrate that loss of C9orf72 function impairs synaptic function at NMJs and result in motor deficits. We postulate that synaptic deficits observed in repeat expansions or DPR models may be the result of an indirect effect related to an impact of the repeats on C9orf72 levels.

2.5 Methods

Zebrafish Husbandry

Adult zebrafish (*Danio rerio*) were maintained at 28°C at a light/dark cycle of 12/12 h in accordance with Westerfield zebrafish book. Embryos were raised at 28.5 °C, and collected and staged as previously described (Kimmel *et al.*, 1995). All the animal experiments were performed in compliance with the guidelines of the Canadian Council for Animal Care and received approval from the INRS-CNBE ethics committee.

Anti-c9orf72 (synthetic miRNA) RNAi target site selection

We first generated a template with c9orf72 RNA sequence, including 5'- and 3'-UTR sequence. 3'-UTR minimal sequence has been obtained from analysis of data available on ensembl (http://asia.ensembl.org/) with zebrafish GRCz11 genome iteration and on Targetscan Fish website (http://www.targetscan.org/fish_62/). We analysed and annotated *c9orf72* sequence for

identifying and avoiding selecting target sequence that would run across i) potential polymorphisms in the 3'UTR sequence and ii) endogeneous miRNA. Based on these data we selected 4x unique target sites on the 3'UTR sequence of *c9orf72* that do not show any off-specific match across the zebrafish genome. Each site and corresponding mature anti-*c9orf72* synthetic miRNA are presented in Supplementary Table 1.

RNAi Plasmid generation

To generate the *c9orf72*-RNAi transgene (Tol2-UBI:dsRED:*c9rof72*-1234-Cryst:eGFP) used in this study to silence the gene *c9orf72*, we first used a previously generated empty RNAi-plasmid compatible with the tol2-kit, pME-RNAi642 (Giacomotto *et al.*, 2015). Based on this design and following previous instructions, we designed 4x anti-*c9orf072* miRNAs stem loops compatible with the pME-RNAi642 (Supplementary Table 1). pME-RNAi642 was digested with BsmBI and gel-extracted. Each stem loops (x4) were annealed and inserted into pME-RNAi642 following previous instructions (Giacomotto *et al.*, 2015). 4x different pME-RNAi-*c9orf72* has been generated and named pME-RNAi-*c9orf72*-1 to -4. We further chained the 4x stem loop. We ended with a 4x-anti-*c9orf72* RNAi pME plasmid named pME-RNAi-*c9orf72*-1234. In parallel, a custom made 1456-pDEST-miniTol2-R4-R2_Cryst:eGFP clone was generated; this clone presents miniTol2 sequence surrounding gateway Att-R4 and Att-R2 sequences followed by a Cryst:eGFP cassette (Crystallin-promoter driving eGFP into lenses for identifying transgenic/carrier fish). Following manufacturer instruction, we performed a Gateway LR-reaction mixing/recombining p5E-Ubi (Ubiquitin promoter) and pME-RNAi-*c9orf72*-1234 into 1456-pDEST-miniTol2-R4-R2_Cryst:eGFP. Final plasmid obtain was named Tol2-UBI:dsRED:*c9rof72*-1234-Cryst:eGFP and used to perform one-cell stage injections for transgene integration.

Injections for transgene integration and rescue experiments

To integrate Tol2-UBI:dsRED:*c9orf72*-1234-Cryst:eGFP construct into the zebrafish genome, 1 nl of a mix of 30 ng/µl of construct and 25 ng/µl of Transposase mRNA was injected into one-cell stage embryos using the Picospritzer III pressure ejector. Rescue experiment was performed using human *C9orf72* mRNA (NM_001256054.3) that was produced via an ORF clone of human *C9orf72* long transcript purchased from GeneCopoeia. *In vitro* transcription was done using T7 message machine kit (Ambion) and 1 nl of *C9orf72* mRNA (100 ng/µl) was injected into the 1-cell stage embryos.

Western Blot

Larvae were collected at 6 dpf fish and for each condition thirty larvae were lysed in 150 µL lysis buffer lysis buffer containing 150 mM NaCl, 50 mM Tris-HCl pH 7.5, 1% triton, 0.1% SDS, 1% sodium deoxycolate, and protease inhibitors cocktail (1:10, Sigma-Aldrich). The lysates were then centrifuged at 10,000 rpm for 10 minutes at 4°C. The supernatant was collected and protein concentration was estimated using Bradford assay (BioRad). Western blotting was performed using 40 µg lysate per sample which were resolved on a 7.5% SDS-polyacrylamide gel (BioRad). After electrophoresis, proteins on the gel were electrotransferred onto PVDF mini-membranes (BioRad). The membranes were blocked with 5% non-fat milk solution in 1X phosphate buffered saline or with 5% bovine serum albumin (Sigma) in 1X Tris buffered saline for immunoblotting with Novus Npb2-15656 (1:5000) antibody against C9orf72 and Sigma A5441 (1:5000) antibody was used against β-actin. Detection was performed using goat anti-rabbit and goat anti-mouse antibodies respectively conjugated with horse radish peroxidase. Bands were visualized with ECL and imaged using ChemiDoc (Biorad). Quantifications were performed with Image Lab (BioRad) normalizing using β-actin.

Gene expression study

RNA was isolated from ~30 embryos using TriReagent® (Sigma) according to manufacturer's protocol. 1µg of RNA was used for cDNA synthesis by SuperScript®Vilo™ kit (Invitrogen). RT-qPCR was run with SYBR Green Master Mix (Bioline) using the LightCycler® 96 (Roche). *ef1a* was used as the reference gene for normalization and following primers were used for *C9orf72*: FW: 5' – GTGTGCCAGAGGAGGTTGAT– 3'; RV:5' – ACAGCTGTCTCCAATATCATCG– 3'.

Gross morphology and survival assessment

Larvae were assessed for their survival rate and morphological phenotypes. The sample sizes for control and C9-miR were as follows: three different batches (N=3) each batch containing 25 larvae (n=25) for both control and C9-miR. Gross morphology was observed under a stereomicroscope (Leica S6E).

GGGGCC expansion repeat microinjections

GGGGCC repeat constructs (p3s and p91s) were kindly provided by Dr. Ludo Van Den Bosch and Dr. Adrian Isaacs. Synthesis of mRNAs and microinjections were performed as previously described (Swinnen *et al.*, 2018).

Behavioural assay

Larvae (6 dpf) were separated into single wells of a 96-well plate containing 200µl of E3 media and habituated in the Daniovision® recording chamber (Noldus) for 1 hour before start of experiment. Larval locomotor activity was monitored over light-dark cycles using the Daniovision® apparatus. Analysis was performed using the Ethovision XT12 software (Noldus) to quantify the total swimming distance in given hours and the locomotor activity per second.

NMJ morphology in larval zebrafish

Immunohistochemical analyses were performed on 2 and 6 dpf zebrafish to visualize NMJ pre- and postsynaptic structures. Briefly, animals were fixed in 4% paraformaldehyde overnight at 4°C. After fixation, the larvae were rinsed several times (1 hour) with PBS-Tween and then incubated in PBS containing 1 mg/ml collagenase (30 minutes for 2 dpf fish and 180 minutes for 6 dpf fish) to remove skin. The collagenase was washed off with PBS-Tween (1 hour), and the larvae were incubated in blocking solution (2% NGS, 1% BSA, 1% DMSO, 1% Triton-X in PBS) containing 10 mg/ml Tetramethylrhodamine conjugated α-bungarotoxin (Thermofisher T1175) for 30 minutes. The larvae were rinsed several times with PBST (30 minutes) and then incubated in freshly prepared block solution containing primary antibody SV2 (1:200, Developmental Studies Hybridoma Bank) overnight at 4°C. Following this, larvae were incubated in block solution containing a secondary antibody (Alexa fluor 488, 1:1,000, cat# A-21042, Invitrogen) for overnight at 4°C. The following day the larvae were washed several times with PBST and mounted on a glass slide in 70% glycerol. Slides were blinded for z-stack imaging with a Zeiss LSM780 confocal microscope (Carl Zeiss, Germany). The images were then processed with ZEN software (Carl Zeiss). Co-localization of pre- and post-synaptic structures per somite were counted per fish from a set of stacked Z-series images using ImageJ (NIH).

TDP-43 immunostaining

Larval zebrafish (6 dpf) were fixed in 4% paraformaldehyde overnight at 4°C. After fixation, the larvae were rinsed several times (1 hour) with PBS-Tween and then incubated in PBS containing 1 mg/ml collagenase (30 minutes for 2 dpf fish and 180 minutes for 6 dpf fish) to remove skin. The collagenase was washed off with PBS-Tween (PBST) (1 hour), and the larvae were incubated in blocking solution (2% NGS, 1% BSA, 1% DMSO, 1% Triton-X in PBS) for 30 minutes. The larvae were rinsed several times with PBS-Tween (30 minutes) and then incubated in block solution containing primary antibody TDP-43 (1:200, Sigma Aldrich) overnight at 4°C. Following this, larvae were incubated in block solution containing a secondary antibody (Alexa fluor 555, 1:1,000, Invitrogen) for overnight at 4°C. The following day the larvae were washed several times with PBST and incubated in Hoechst solution for 15 minutes. Larvae were then washed in PBST and mounted in glycerol. Z-stack images were taken using a Zeiss LSM780 confocal microscope (Carl Zeiss, Germany). The images were then processed with ZEN software (Carl Zeiss). Images were acquired using the same settings. Researcher was blinded to genotype prior to image analysis. Each image was processed using Fiji (ImageJ) software and analysis was performed in the skeletal muscles in 50µm by 50µm areas. A threshold was used to define the nuclei area and cytoplasm was defined as outside the nuclei. ImageJ Plugin versatile ward tool was used to detect nucleus and the remaining large area devoid of nuclei was considered as the cytoplasm. TDP-43 mean intensity was measured in these two areas. The ratios were then analysed in GraphPad Prism6 and a Kruskall-Wallis test was performed to calculate the statistical significance.

Hematoxylin & Eosin staining

For Hematoxylin and Eosin staining, spinal cord sections (15µm) sections of adult fish were stained with Hematoxylin (Statlab) for 4 min, and washed with alcohol-acid, and were rinsed with tap water. The sections were then soaked in saturated Lithium Carbonate solution for 10 sec, and then rinsed it with tap water. Finally, staining was performed with Eosin Y (Statlab) for 2 min, and mounted under coverslip with permount mounting media.

Neuromuscular junction staining on adults

Adult wild-type and C9-miR zebrafish were euthanized and fixed in 4% paraformaldehyde for 72h at 4°C. The fish were then embedded in paraffin and longitudinal sections (15µm) were obtained on a Leica microtome and process for immunostaining. Slides with the sections were incubated

xylene twice and followed by rehydration in 4 successive baths of 100%, 95%, 70% and 50% ethanol in distilled water respectively. Sections were blocked for 30 minutes in with blocking solution (2% NGS, 1% BSA, 1% DMSO, 1% Triton-X in PBS) containing 10 mg/ml Tetramethylrhodamine conjugated α-bungarotoxin (Thermofisher T1175) to label postsynaptic acetylcholine receptors. Sections were then rinsed several times with PBST and then incubated with primary antibody SV2 (1:200, Developmental Studies Hybridoma Bank) overnight at 4°C. After washing, sections were incubated in block solution containing a secondary antibody (Alexa fluor 488, 1:1,000, cat# A-21042, Invitrogen) for 2h, followed by rising and mounting. Slides were blinded for z-stack imaging with a Zeiss confocal microscope and the images were then processed with ZEN software (Carl Zeiss). Differences in NMJ integrity were determined by examining SV2 and αBTX co-localization per muscle fiber on each slice section per fish to calculate the number of innervated junctions using ImageJ.

Motoneuron staining and TDP-43 pathology in adult fish

Adult wild-type and C9-miR zebrafish were euthanized and fixed in 4% paraformaldehyde for 72h at 4°C. The fish body trunk was crossed-sectioned using a microtome at 15μm thick slices. Sections were incubated xylene twice and followed by rehydration in 4 successive baths of 100%, 95%, 70% and 50% ethanol in distilled water respectively. They were then rinsed several times in PBS and incubated in citrate buffer (1 M, pH 6) for antigen retrieval. After several washes in PBST, sections were incubated in blocking buffer (1% NGS, 0.4% Triton-X) for 1h, followed by incubation in primary antibody ChAT (1:500; Invitrogen) alone or with primary antibody TDP-43 (1:200, Sigma Aldrich) at 4°C overnight. The next day, sections were washed and incubated in blocking solution containing secondary antibody Alexa Fluor 488 (1:750; Molecular Probes, Invitrogen) for 2h, followed by rinsing and mounting in Prolong Gold antifade reagent with DAPI (Invitrogen). Motor neurons in spinal cord were identified as ChAT-positive objects greater than 10 μm per section (Ramesh *et al.*, 2010). Researcher was blinded to genotype during quantification and analysis. Motor neurons in spinal cord cross-sections were quantified using Fiji ImageJ (NIH). For assessment of TDP-43 pathology, the ROI manager tool in Fiji ImageJ was used to define ChAT+ motor neurons in TDP-43-stained sections and the colocalization between the cellular TDP-43 antibody signal and the nuclear DAPI stain in these neurons were measured using Fiji ImageJ and analyzed.

FM1-43 staining

Zebrafish larvae (6dpf) were first anesthetized in Evan's solution (134 mM NaCl, 2.9 mM KCl, 2.1 mM $CaCl_2$, 1.2 $MgCl_2$, 10 mM HEPES, 10 mM glucose) containing 0.02% tricaine (Sigma Aldrich). The larvae were then pinned to a Sylgard coated dish both at the head and extreme tail end using electrolytically sharpened tungsten needles. The skin was then carefully peeled away to expose the muscles and to permit access to FM1-43 (Molecular Probes). The fish were treated with Evan's solution containing 10 μM of FM1-43 to allow preloading penetration of the dye molecules. After 10 min, the fish were transferred to a high potassium Hank Buffer Salt Solution (HBSS) (97 mM NaCl, 45 mM KCl, 1 mM $MgSO_4$, 5 mM HEPES, 5 mM $CaCl_2$) containing 10 μM of FM1-43 for 5 minutes. The fish were then tranferred in Evan's solution with 10 μM of FM1-43 finished for an additional 3 minutes, after which loading was complete. The larvae were then washed with a low calcium Evan's solution (0.5 mM $CaCl_2$) three times for 5 minutes to minimize spontaneous release of loaded synaptic vesicles. The fish were imaged for FM1-43 staining using a 40X Examiner A1 microscope (Zeiss). Blind measurements of FM1-43 staining at NMJs in wild-type control and C9-mIR fish were performed per 3 somites per fish for each genotype using Fiji ImageJ (NIH).

Electrophysiology recordings

Whole-cell patch clamp recordings were taken from muscle cells of 6 dpf larvae. The preparation was bathed in an extracellular solution (134 mM NaCl, 2.9 mM KCl, 1.2 mM MgCl, 10 mM HEPES, 10 mM glucose, pH 7.8) containing 1 μM of tetrodotoxin (TTX; Tocris, UK) in order to block action potentials during mEPC recordings. Patch clamp electrodes (2-4 MΩ) were filled with an intracellular solution (130 mM CsCl, 8 mM NaCl, 2 mM $CaCl_2$, 10 mM HEPES, 10 mM EGTA, 4 Mg-ATP, 0.4 Li-GTP, pH 7.4). Miniature endplate currents from white muscle fibers were recorded in the whole-cell configuration with an Axopatch 200B amplifier (Molecular Devices) at a holding potential of -60 mV, low-pass filtered at 5 kHz and digitized at 50 kHz. Series resistance was compensated by at least 85% using the amplifier's compensation circuitry. Synaptic currents were acquired using the pCLAMP10 software (Molecular Devices).

Analysis of miniature endplate currents

Miniature endplate currents (mEPCs) were analyzed using the AxoGraph X software. The mEPC recordings were examined by the software, and synaptic events were detected using a template

function. Overlapping or misshapen events were removed and the remaining events were averaged and the properties (amplitudes, decay time constants and frequencies) of the averaged trace were measured. Events with slow rise times and low amplitudes were excluded from the analysis, therefore, only fast rise time events were included in our analysis since these events originated from the cells that were patch clamped rather than from nearby, electrically-coupled muscles. Single decay time constants were fit over the initial (fast) decay portion and over the distal (slow) portion of the decay. For each *n*, currents were recorded from a single white muscle fiber from a single larva. Zebrafish twitch white fibers make up the bulk of the trunk musculature and they are easily identifiable under the microscope. We focused on white fibers as mammalian skeletal musculature is mostly comprised of twitch fiber types.

Rab3a immunostaining in larval zebrafish

Larval zebrafish (6 dpf) were fixed in 4% paraformaldehyde overnight at 4°C. After fixation, the larvae were rinsed several times (1 hour) with PBS-Tween and then incubated in PBS containing 1 mg/ml collagenase (30 minutes for 2 dpf fish and 180 minutes for 6 dpf fish) to remove skin. The collagenase was washed off with PBS-Tween (1 hour), and the larvae were incubated in blocking solution (2% NGS, 1% BSA, 1% DMSO, 1% Triton-X in PBS) for 30 minutes. The larvae were rinsed several times with PBST (30 minutes) and then incubated in freshly prepared block solution containing primary antibody Rab3a (1:100, Sigma Aldrich, cat#WH0005864M1) overnight at 4°C. Following this, larvae were incubated in block solution containing a secondary antibody (Alexa fluor 488, 1:1,000, Invitrogen) for overnight at 4°C. The following day the larvae were washed several times with PBST and mounted on a glass slide in 70% glycerol. Z-stack images were taken using a Zeiss LSM780 confocal microscope (Carl Zeiss, Germany). The images were then processed with ZEN software (Carl Zeiss). Researcher was blinded to genotype prior to image analysis. The number and area of Rab3a-positive puncta per three somites were counted and measured per fish using Fiji ImageJ (NIH).

Mass spectrometry sample preparation

Proteins were extracted with the protocol used for western blot protein extraction. Then, a 1:8:1 ratio was used to precipitate proteins, 1X of cell lysates, 8X of 100% ice cold acetone and 1X of 100% trichloroacetic acid in low binding protein tubes. 20 µg of proteins were precipitated.

Proteins were incubated at -20°C for 12 hours and centrifuged at 11,500 rpm for 15 minutes at 4°C. Supernatant was then discarded.

A standard TCA protein precipitation was first performed to remove detergents from the samples (or acetone precipitation). Protein extracts were then re-solubilized in 10 µL of a 6M urea buffer. Proteins were reduced by adding 2.5 µL of the reduction buffer (45 mM DTT, 100 mM ammonium bicarbonate) for 30 min at 37ºC, and then alkylated by adding 2.5 µL of the alkylation buffer (100 mM iodoacetamide, 100 mM ammonium bicarbonate) for 20 min at 24ºC in dark. Prior to trypsin digestion, 20 µL of water was added to reduce the urea concentration to 2M. 10 µL of the trypsin solution (5 ng/µL of trypsin sequencing grade from Promega, 50 mM ammonium bicarbonate) was added to each sample. Protein digestion was performed at 37ºC for 18 h and stopped with 5 µL of 5% formic acid. Protein digests were dried down in vacuum centrifuge and stored at -20 ºC until LC-MS/MS analysis.

Mass spectrometry (LC-MS/MS)

Prior to LC-MS/MS, protein digests were re-solubilized under agitation for 15 min in 10 µL of 0.2% formic acid. Desalting/cleanup of the digests was performed by using C18 ZipTip pipette tips (Millipore, Billerica, MA). Eluates were dried down in vacuum centrifuge and then re-solubilized under agitation for 15 min in 12 µL of 2%ACN / 1% formic acid. The LC column was a PicoFrit fused silica capillary column (15 cm x 75 µm i.d; New Objective, Woburn, MA), self-packed with C-18 reverse-phase material (Jupiter 5 µm particles, 300 Å pore size; Phenomenex, Torrance, CA) using a high pressure packing cell. This column was installed on the Easy-nLC II system (Proxeon Biosystems, Odense, Denmark) and coupled to the Q Exactive (ThermoFisher Scientific, Bremen, Germany) equipped with a Proxeon nanoelectrospray Flex ion source. The buffers used for chromatography were 0.2% formic acid (buffer A) and 100% acetonitrile/0.2% formic acid (buffer B). Peptides were loaded on-column at a flowrate of 600 nL/min and eluted with a 2 slope-gradient at a flowrate of 250 nL/min. Solvent B first increased from 2 to 40% in 120 min and then from 40 to 80% B in 20 min. LC-MS/MS data was acquired using a data-dependant top17 method combined with a dynamic exclusion window of 7 sec. The mass resolution for full MS scan was set to 60,000 (at m/z 400) and lock masses were used to improve mass accuracy. The mass range was from 360 to 2000 m/z for MS scanning with a target value at 1e6, the maximum ion fill time (IT) at 100 ms, the intensity threshold at 1.0e4 and the underfill ratio at 0.5%. The data dependent MS2 scan events were acquired at a resolution of 17,500 with the maximum ion fill time at 50 ms and the target value at 1e5. The normalized collision energy used

was at 27 and the capillary temperature was 250ºC. Nanospray and S-lens voltages were set to 1.3-1.7 kV and 50 V, respectively.

The peak list files were generated with Proteome Discoverer (version 2.3) using the following parameters: minimum mass set to 500 Da, maximum mass set to 6000 Da, no grouping of MS/MS spectra, precursor charge set to auto, and minimum number of fragment ions set to 5. Protein database searching was performed with Mascot 2.6 (Matrix Science) against the Refseq Danio Rerio protein database. The mass tolerances for precursor and fragment ions were set to 10 ppm and 0.1 Da, respectively. Trypsin was used as the enzyme allowing for up to 1 missed cleavage. Cysteine carbamidomethylation was specified as a fixed modification, and methionine oxidation as variable modifications. Data analysis was performed using Scaffold (version 4.8).

Plasmid Constructions

Human *C9orf72* cDNA (Genecopoeia) was cloned into pmRFP-C1 plasmid (Addgene, #54764) to generate RFP-C9orf72 plasmids. Myc-SMCR8 and Myc-SV2a plasmids were purchased from Genecopoeia.

Cell transfection, Immunoprecipitation and Western Blotting

HEK293T cells were co-transfected with 4 µg of RFP-C9orf72 or RFP (as negative control) and 4 µg of Myc-SMCR8 or Myc-SV2a plasmids using polyethylenimine. The cells were rinsed 3x with cold PBS before being lysed with 1mL of RIPA buffer (0.01M PBS, 0.0027M KCl, 0.137M NaCl, $0.1\%_{v/v}$ SDS, $0.5\%_{m/v}$ Na-deoxycholate, 1% NP-40, pH 7.4) with protease inhibitors for 15 min on ice. The RIPA was then collected into microcentrifuge tubes and centrifuged at 21,000g for 15 min at 4°C. The supernatant was then split into input and RFP-Trap fractions. 50 µL of lysate was mixed with 50 µL of 2x Lämmli buffer and stored at -20°C. The remaining lysate was mixed with 20 µL of RFP-Trap agarose beads (twice rinsed with 500 µL RIPA buffer) and incubated for 2 hours. The beads were then rinsed 5 times with RIPA buffer before the samples were resuspended in 60 µL of 1x Lämmli buffer. The samples with SMCR8 were then denatured at 94°C for 5 minutes while samples with the transmembrane SV2a partner were left at room temperature.

20 µL of the samples were then loaded into a 7.5% gel for SDS-PAGE at 150V for 1 hour, before being transferred to a PVDF membrane for 30 minutes in a BioRad Turboblot. The membranes were then placed in blocking solution (5% milk powder in PBS-T) before being incubated overnight

with rabbit anti-C9orf72 (1:1000; Abcam #ab221137) or mouse anti-Myc antibodies (1:1000; Sigma #M5546). The membranes were then rinsed 3 times with 10mL PBS-T before being incubated at room temperature in 10 mL of horseradish peroxidase-conjugated secondary antibodies (HRP goat anti-rabbit or HRP goat anti-mouse; Sigma). The membranes were then rinsed 3 times in 10 mL PBS-T before ECL solution (BioRad) was added to the membranes for imaging on a BioRad imager. The imaged blots were quantified using ImageLab.

Statistics and reproducibility

All zebrafish experiments were performed on at least three replicates (N) and each consisted of a sample size (n) of 5-65 fish. Data are presented as Mean±SEM. Significance was determined using either Student's t-test or One-way ANOVA followed by multiple comparisons test. A Tukey post-hoc multiple comparisons test was used for normally distributed and equal variance data. Kruskal-Walllis ANOVA and Dunn's method of comparison were used for non-normal distributions. All graphs were plotted using the Graphpad PRISM software. Significance is indicated as * $p<0.05$, ** $p<0.001$ and *** $p<0.0001$.

Conflicts of interest

The authors declare no competing interests.

Data Availability

Data represented in this manuscript are stored on hard-drives for permanent storage and on the cloud. These data as well as the material used in this study will be available on request.

2.6 Acknowledgments

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Project Grant (APP1165850) and a Rebecca L. Cooper Medical Research Project Grant (PG2019405). The authors would like to summer student Gabrielle Fortier for her assistance in some of the zebrafish experiments.

2.7 Supplemental data

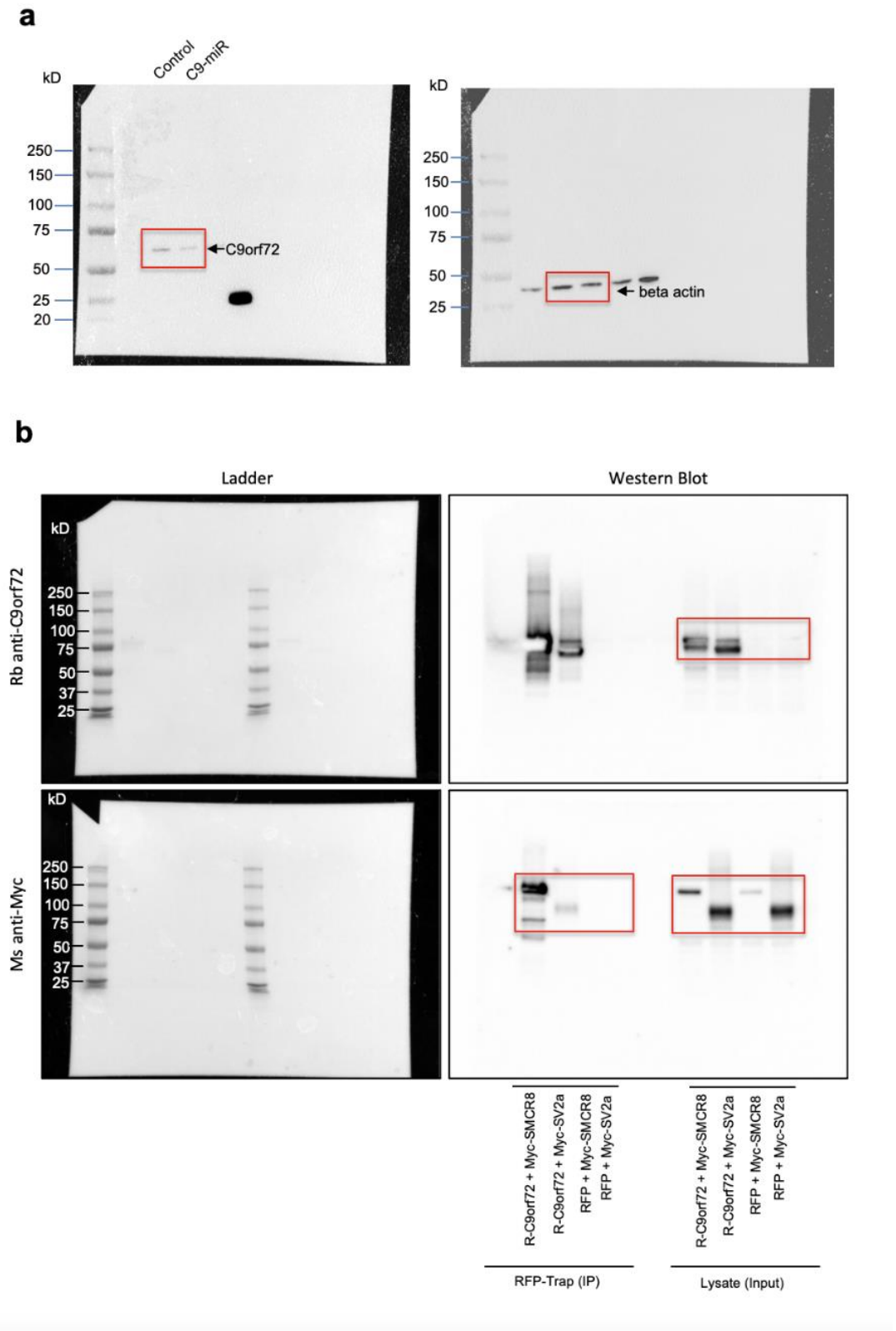

Figure 2-9: Full Western Blot for (a) Figure 1d and (b) Figure 7c.

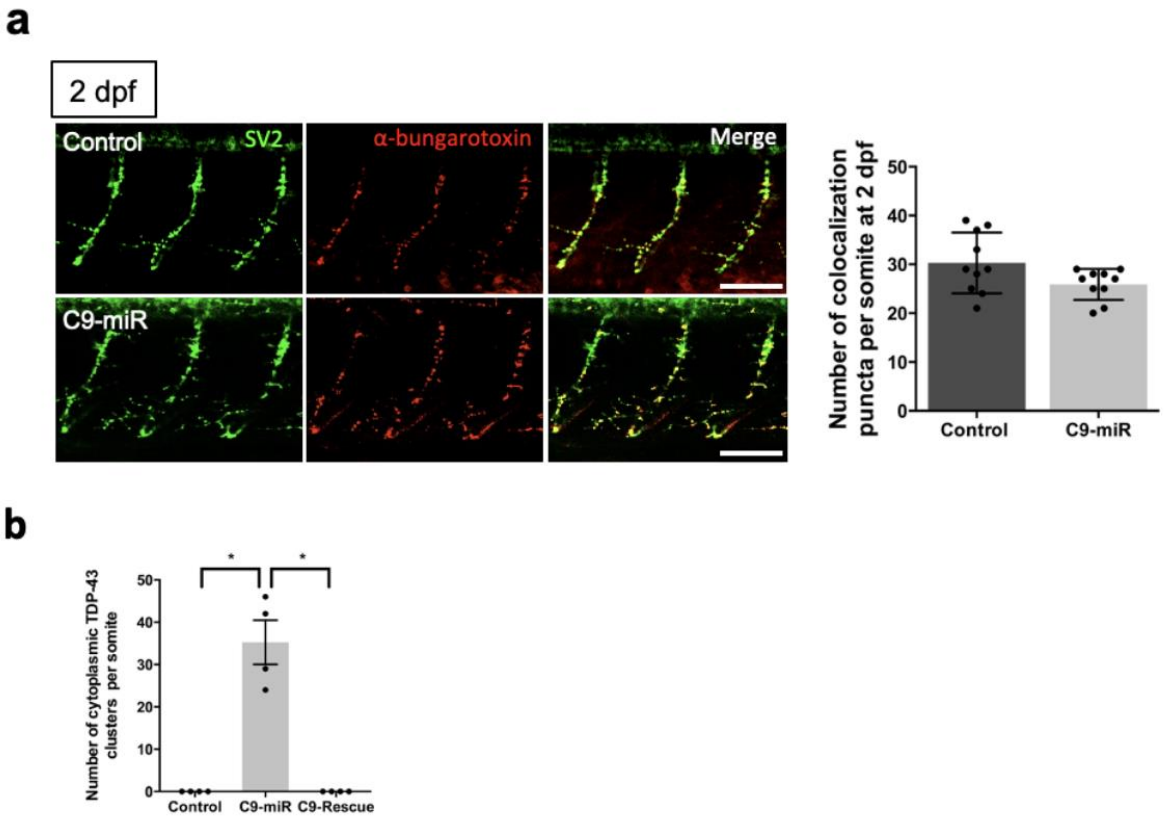

Figure 2-10: Characterization of C9-miR fish.

a. Representative images of co-immunostaining of zebrafish neuromuscular junctions with presynaptic (SV2) and postsynaptic (α-bungarotoxin) markers in 2 dpf zebrafish. Scale bar = 50 μm. Quantification Of the colocalizing pre- and post-synaptic markers per somite showed no differences between C9-miR and controls at early embryonic stages (2 dpf; n=10; p=0,064; Student's t-test). b. Quantification of cytoplasmic TDP-43 clusters in control, C9-miR and C9-rescue fish (n=4; *p<0,05; One-way ANOVA). Data presented as mean ±SEM. n represents number of fish.

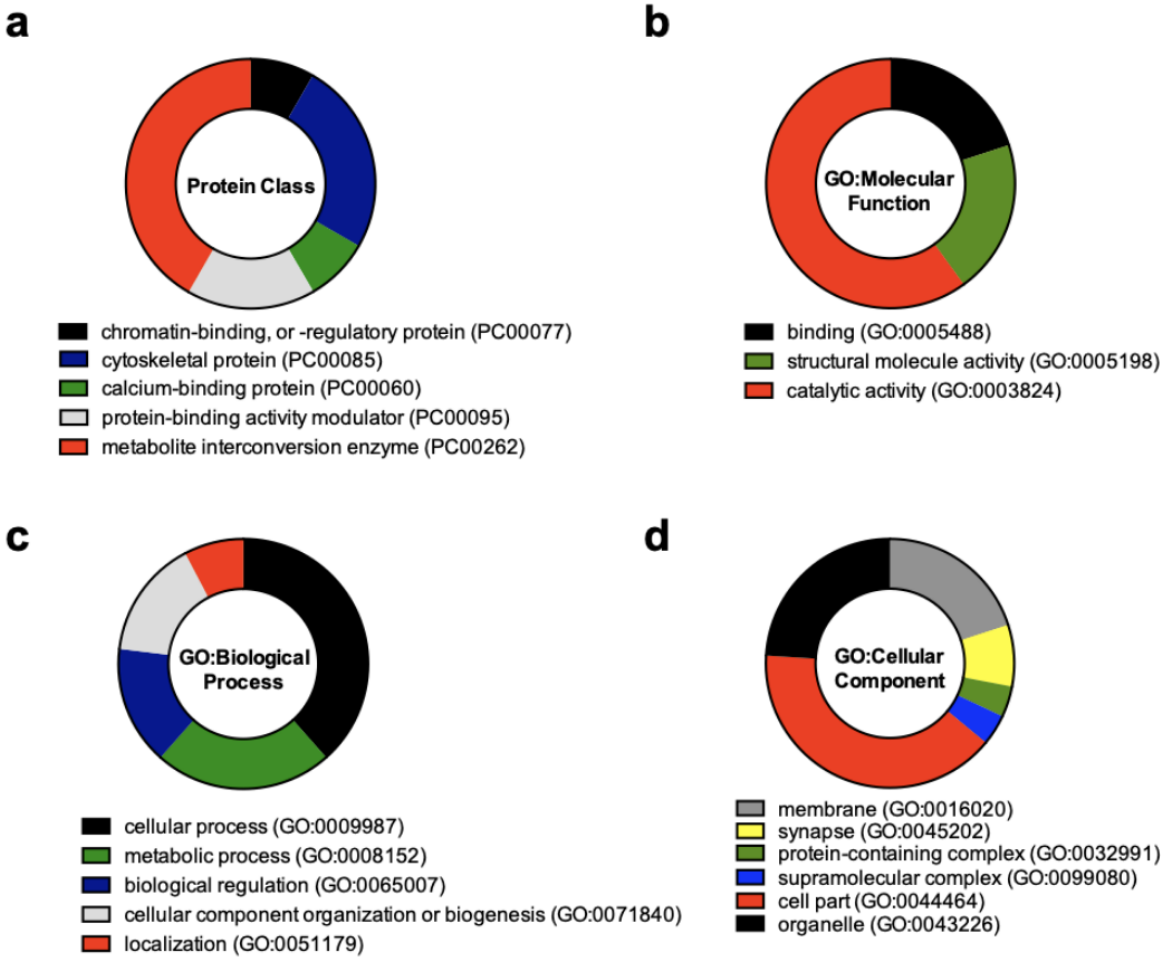

Figure 2-11: Proteomic analysis in C9-miR and control larvae.

Protein class (a), Molecular function (b), Biological processes (c) and cell component (d). GO-term classifications that are enriched in the differentially expressed proteins.

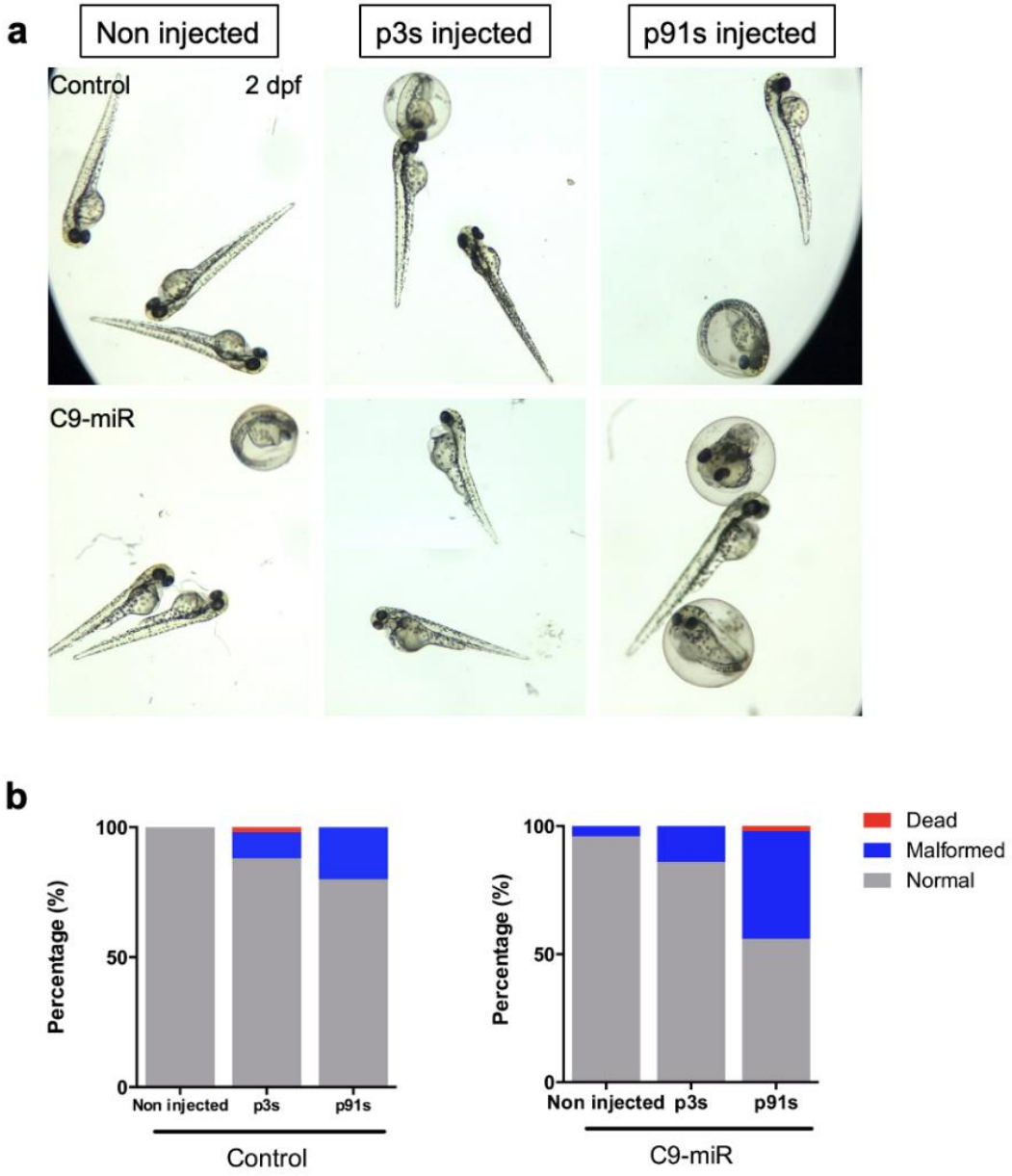

Figure 2-12: Expression of long GGGGCC repeats exacerbates toxicity in C9-miR fish.

a. Images if the control and C9-miR zebrafish 2 dpf fish uninjected or injected with GGGGCC short (p3s) andlong (p91s) mRNA. b. Expressing p91s mRNA in C9-miR increases toxicity (right panel; Chi-square=53,72, df=4, N=3, n=75) compared to control (left panel). N represents number of experimental repeats and n represents number of fish.

Table 2-1: Design of 4x anti-C9orf072 miRNAs stem loops compatible with the pME-RNAi642.

NAME	SEQUENCE
C9ORF72-01 mature miR (5'-3')	ACATCAAGACGCAAGAGTGTG
C9ORF72-02 mature miR (5'-3')	TGCAGAATGCTGTAAACAAAG
C9ORF72-03 mature miR (5'-3')	TTCATAAACCTCAAGACCACA
C9ORF72-04 mature miR (5'-3')	CTATTAGTCAACAAGCCGTTA
C9ORF72-01 star miR (5'-3')	CACACTCTCGTCTTGATGT
C9ORF72-02 star miR (5'-3')	CTTTGTTTAGCATTCTGCA
C9ORF72-03 star miR (5'-3')	TGTGGTCTAGGTTTATGAA
C9ORF72-04 star miR (5'-3')	TAACGGCTTTGACTAATAG
C9ORF72-01a stem loop	TGCTGACATCAAGACGCAAGAGTGTGGTTTTGGCCACTGACTGACCACACTCTCGTCTTGATGT
C9ORF72-01b stem loop	CCTGACATCAAGACGAGAGTGTGGTCAGTCAGTGGCCAAAACCACACTCTTGCGTCTTGATGTC
C9ORF72-02a stem loop	TGCTGTGCAGAATGCTGTAAACAAAGGTTTTGGCCACTGACTGACCTTTGTTTAGCATTCTGCA
C9ORF72-02b stem loop	CCTGTGCAGAATGCTAAACAAAGGTCAGTCAGTGGCCAAAACCTTTGTTTACAGCATTCTGCAC
C9ORF72-03a stem loop	TGCTGTTCATAAACCTCAAGACCACAGTTTTGGCCACTGACTGACTGTGGTCTAGGTTTATGAA
C9ORF72-03b stem loop	CCTGTTCATAAACCTAGACCACAGTCAGTCAGTGGCCAAAACTGTGGTCTTGAGGTTTATGAAC
C9ORF72-04a stem loop	TGCTGCTATTAGTCAACAAGCCGTTAGTTTTGGCCACTGACTGACTAACGGCTTTGACTAATAG
C9ORF72-04b stem loop	CCTGCTATTAGTCAAAGCCGTTAGTCAGTCAGTGGCCAAAACTAACGGCTTGTTGACTAATAGC

Table 2-2: Proteomic analysis revealed significant differential expression of 24 proteins.

LOG2 (Fold Change)	Identified Proteins	Gene name	p-value
-4.128008727	synaptic vesicle glycoprotein 2A	sv2a	0.02070914
-3.654641415	calpastatin	cast	0.0416028
-3.128015343	hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (soluble) (AAH49456.1)	hmgcs1	0.02950878
-2.863542157	Alkaline phosphatase	alpl	0.03635185
-2.737198494	Methylenetetrahydrofolate dehydrogenase (NADP+ dependent)	mthfd2	0.02270433
-2.404288943	L-2-hydroxyglutarate dehydrogenase, mitochondrial	l2hgdh	0.04519278
-2.391186305	integrin alpha-2-like isoform X1	itga2.2	0.03836482
-2.246910845	adapter molecule crk	crk	0.04630673
-2.077591185	complement C5	c5	0.03424657
-2.054375027	Oxct1b protein	oxct1b	0.04522116
-1.810816378	dmX-like protein 2	dmxl2	0.04981243
-1.791570505	uncharacterized protein LOC550458 isoform 1 [Danio rerio]	tubb6	0.04704342
1.824214917	fast skeletal myosin light chain 3	mylz3	0.0492682
1.993865177	myosin, light chain 1, alkali; skeletal, fast	myl1	0.04369252
2.254058278	Apolipoprotein A-IV	apoa4b.1	0.0309075
2.28305222	cytochrome c oxidase subunit 7A2 like	cox7a2l	0.04688909
2.440741318	Enolase 3, (beta, muscle)	eno3	0.02544621
2.500419058	serine/threonine-protein phosphatase 2A catalytic subunit beta isoform	ppp2cb	0.04183156
2.538029927	uncharacterized protein si:dkey-9l20.3	si:dkey-9l20.3	0.0401815
2.662770409	uncharacterized protein LOC768128	cst14b.1	0.02160492
2.986970782	nonhistone chromosomal protein HMG-14A-like	hmgn7	0.0220809
3.182978967	aspartate--tRNA ligase, cytoplasmic	dars1	0.03100769
3.239143855	Cluster of Zgc:112374 [Danio rerio] (AAI64397.1)	pon3.1	0.04934178
3.709872848	parvalbumin 8	pvalb8	0.0393578

3 CALPASTATIN COMPENSATION RESCUES NEUROMUSCULAR DYSFUNCTION IN A C9ORF72 ALS LOSS OF FUNCTION MODEL

This chapter is composed of a paper in preparation.

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Contribution: SAP and ZB conceived the work. ZB designed, collected, analyzed and interpreted the results. AP collected human tissues and provided access to the CRCHUM biobank. ZB and SAP drafted and prepared the final version of the manuscript.

3.1 Abstract

Amyotrophic lateral sclerosis (ALS) is a rapidly progressing, fatal neurodegenerative disease with no effective treatment. Identification of novel therapeutic targets and treatments are desperately needed for ALS. Calpastatin has been reported to have a neuroprotective effect in various neurodegenerative disorders. We previously developed a zebrafish C9orf72 ALS model that replicates hallmarks of ALS including neuromuscular deficits and TDP-43 pathology. Calpastatin is significantly reduced in expression in this zebrafish ALS model. We then examine the effects of restoring calpastatin function with two compounds, Calpeptin and calpastatin active peptide, on neuromuscular dysfunction in this zebrafish C9orf72 ALS model. Our analysis of the locomotor behaviour reveals an improvement of the fish motor function and the assessment of the neuromuscular junctions show preservation of their anatomical features. Additionally, both synaptic vesicle turnover and release of quantal synaptic vesicles at the NMJ are restored upon treatment with the two compounds. Altogether, these results suggest that calpastatin may be a potential therapeutic target for stabilizing and preserving neuromuscular function in ALS.

3.2 Introduction

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by a motoneuron degeneration and loss leading to patient paralysis and death within the five years after the diagnosis (Andersen *et al.*, 2011). Considerable progress has been made in unraveling the genetic etiology of ALS, with the identification of more than two dozen genes associated with the disease manifestation (Ghasemi *et al.*, 2018; Talbott *et al.*, 2016). An expansion in the first intronic region of the gene C9orf72 is the most common genetic cause of the disease (DeJesus-Hernandez *et al.*, 2011; Renton *et al.*, 2011). Current approved treatments for ALS only have mild beneficial effects. Therefore, there is an urgent need for developing and assessing new therapeutics for ALS. Defects in neuromuscular junctions (NMJs) occur at early stages of the disease, thus stabilizing and preserving NMJs represents a potential therapeutic strategy to slow/halt ALS disease progression.

Calpastatin/calpain system has been implicated in several neurodegenerative diseases (de la Fuente *et al.*, 2020; Diepenbroek *et al.*, 2014; Menzies *et al.*, 2015; Rao *et al.*, 2016; Rao *et al.*, 2014; Rao *et al.*, 2008). Calpain activity is regulated by its endogenous inhibitor calpastatin. Calpastatin has four inhibitory domains which confer to it the ability to bind an inhibit four calpain molecules at a time (Hanna *et al.*, 2008; Wendt *et al.*, 2004). Alterations in calcium homeostasis result to pathological calpain activity affecting negatively the function of neurons (Liu *et al.*, 2008; Vosler *et al.*, 2008) and this is accompanied by calpastatin depletion (Rao *et al.*, 2008). In slow channel congenital myasthenic syndrome (SCS), overactive calpain activity leads to impaired synaptic transmission at the NMJ (Groshong *et al.*, 2007). Expression of human calpastatin in a mouse model of SCS restored calpain level, preserved NMJ structure and miniature endplate current and improved synaptic transmission at NMJs (Groshong *et al.*, 2007). Altered calcium homeostasis and synaptic dysfunction have been reported in ALS patients (Fogarty, 2019; Van Den Bosch *et al.*, 2006). However, the potential of calpastatin in protecting NMJ function in ALS has not been explored yet.

Several studies have shown the neuroprotective role of calpastatin in neurodegenerative diseases. For instance, in Huntington's disease, Hu et al demonstrated that inhibiting calpastatin degradation with the small molecule CHIR99201 can prevent calpain cleavage of mutant huntingtin. This in turn, prevented mutant huntingtin aggregation, thus promoting its clearance using the autophagy pathway and increasing neuronal survival in Huntington's disease patient-derived neurons (Hu *et al.*, 2021). In spinal muscular atrophy (SMA) mice model, treatment with a calpain inhibitor, calpeptin, increased levels of SMN and calpastatin in spinal motor neurons (de

la Fuente *et al.*, 2020). In the hSOD1^{G93A} ALS mouse model, calpain levels have been found to be increased and calpastatin level decreased in the mice spinal cord (Rao *et al.*, 2016). Overexpression of calpastatin in hSOD1^{G93A} mice could prevent motoneuron death and promote their survival even at later stage of the disease (Rao *et al.*, 2016). It is currently unknown whether restoring calpastatin function remains a potential effective therapeutic strategy in other genetic forms of ALS such C9orf72 ALS.

In this study, we show that calpastatin expression is decreased in a C9orf72 zebrafish ALS model. We report pharmacologically mimicking calpastatin function with the calpain inhibitor, calpeptin, and a calpastatin active peptide in this ALS model has beneficial effects. In particular, we observe improvement in motor behaviour, NMJ structure, unitary synaptic transmission and synaptic vesicle cycle at NMJs. The effectiveness of compounds targeting the calpastatin system in this zebrafish C9orf72 ALS suggests new potential therapeutics for the treatment NMJ dysfunction in ALS.

3.3 Results

3.3.1 Calpastatin expression is decreased in a C9-LOF model

We recently showed that a zebrafish C9orf72 loss of function (LOF) model recapitulates ALS phenotypes, including motor deficits, muscle atrophy and TDP-43 pathology (Butti *et al.*, 2021). Importantly, proteomic analysis revealed in the zebrafish LOF model (referred hereafter as C9-miR as in Butti *et al.*, 2021) a significant reduction in the expression of calpastatin (*cast*) (Butti *et al.*, 2021). CAST depletion has been reported in several neurodegenerative diseases including Alzheimers, Parkinson, Hungtinton's and restoring CAST levels in model of these diseases (Diepenbroek *et al.*, 2014; Menzies *et al.*, 2015; Rao *et al.*, 2014; Rao *et al.*, 2008) and in hSOD1^{G93A} mice (Rao *et al.*, 2016) has shown to be neuroprotective. We thus sought to confirm the decrease in the expression of *cast* in zebrafish C9-miR compared to wild-type controls. By using a RT-qPCR technique, we observed a marked reduction in the level of *cast* expression in C9-miR larvae compared to controls (Fig 1a).

3.3.2 Altered calpastatin localization in ALS patients

Calpastatin controls calpain activity and alterations in calpain/calpastatin system have been reported in several neurodegenerative diseases. In ALS, altered calpastatin/calpain system has been shown in SOD1 ALS mice (Wootz *et al.*, 2006). Several ALS proteins (TDP-43, C9orf72,

matrin-3, VCP and FUS) are substrates of calpain (De Marco *et al.*, 2022) and prolonged calpain activity has been suggested to be harmful for motor neurons (Stifanese *et al.*, 2014). Overexpression of calpastatin in hSOD1^{G93A} mice lowered calpain activation and improved motor axon loss and their survival (Rao *et al.*, 2016). Prolonged calpain activation also promotes degradation of calpastatin. Changes in cellular localization is a marker of its activation in inhibiting calpain activity (Averna *et al.*, 2003; De Tullio *et al.*, 1999). Calpastatin resides near the nuclear invaginations and when intracellular concentration of Ca^{2+}, calpastatin undergoes an intracellular redistribution to the cytosol to inhibit calpain activity (De Tullio *et al.*, 1999). To gain more insight in calpastatin function in ALS, we examined its cellular localization in healthy (N=2) and ALS individuals' (N=3) motor cortex. Surprisingly, we observed that in ALS patients, calpastatin was strongly expressed in the nucleus compared to controls (Fig. 1b). This finding suggests that there is an altered cellular distribution of calpastatin in ALS motor cortex and that calpastatin inhibitory function on calpain likely occur less in ALS.

3.3.3 Calpastatin active peptide and calpeptin ameliorate motor behavioral phenotype in C9-miR zebrafish

Given the findings about the neuroprotective role of calpastatin in several models of neurodegenerative diseases (de la Fuente *et al.*, 2020; Hu *et al.*, 2021; Rao *et al.*, 2016) and the altered localization of calpastatin in ALS motor cortex, we thus next sought to examine the potential therapeutic effects of restoring calpastatin function in C9-miR fish.

At 6dpf, zebrafish C9-miR larvae exhibit reduced motor activity that could be rescued by overexpression of mRNA encoding the human *C9orf72* long transcript (Butti *et al.*, 2021). Such an impaired motor behaviour in C9-miR zebrafish is consistent with findings that we and others have reported in several other zebrafish models of ALS (Da Costa *et al.*, 2014; Patten *et al.*, 2017; Sakowski *et al.*, 2012; Shaw *et al.*, 2018). Capitalizing on this strong motor phenotype as drug screen readout (Patten *et al.*, 2017), we investigated the potential neuroprotective effects of Calpeptin (Calp- a calpain inhibitor) or calpastatin active peptide (CAST) in C9-miR fish (Fig. 1c). We tested Calp and CAST in C9-miR zebrafish and observed that C9-miR zebrafish treated both Calp and CAST displayed a significant improvement in their motor behavior (Fig. 1d).

3.3.4 Calpeptin (Calp) and calpastatin active peptide (CAST) treatments stabilize NMJ structure and function in C9-miR fish

We next examined whether Calp and CAST treatments were effective in preserving NMJ structure integrity in C9-miR zebrafish. Immunostaining of NMJ structure in 6 dpf zebrafish C9-miR and control larvae with the pre-synaptic marker SV2 and post-synaptic revealed structural abnormalities in C9-miR fish compared to controls (Fig. 2a,b), as we previously reported (Butti *et al.*, 2021). Calp and CAST treatments had no effect on control fish. On the other hand, both compounds had a protective effect on NMJ structure in C9-miR larvae (Fig. 2a,b). This suggest that calpastatin compensation could protect NMJ structure integrity in C9-miR zebrafish.

We next investigated whether ameliorations in NMJ integrity in C9-miR with Calp and CAST treatments also improved synaptic transmission at NMJs by recording and analyzing quantal events (spontaneous miniature end plate currents (mEPCs)) at the NMJs (Fig. 3a). Analysis of mEPCs revealed a significant decrease in amplitude (Fig. 3b) and frequency (Fig. 3c) of mEPCs occurring in C9-miR larvae compared to controls. Treatment with Calp and CAST significantly increased the amplitude (Fig. 3b) and frequency (Fig. 3c) of mEPCs occurring in C9-miR. We did not observe any differences in the rise time (Fig. 3d) or decay time constant (Fig. 3e) kinetics in C9-miR and control with or without Calp and CAST treatments. Altogether, our findings suggest that pharmacological compensation of calpastatin function stabilizes NMJ deficits in a C9orf72 ALS loss of function zebrafish model.

3.3.5 Synaptic vesicle release and recycling is improved in C9-miR fish upon treatment with Calp and CAST

The synaptic protein SV2a is an important component of active zones and synaptic release machinery. We recently reported a downregulation of the synaptic vesicle SV2a in C9-miR zebrafish and a subsequent reduction in the rate of synaptic vesicle release and recycling (Butti *et al.*, 2021). Expression of human calpastatin increases the expression of SV2a and significantly restores impaired neuromuscular transmission in a mouse model of SCS (Groshong *et al.*, 2007; Zhu *et al.*, 2013). We thus sought to further assess synaptic activity at the NMJ in C9-miR upon Calp and CAST treatments by measuring synaptic vesicle cycling using the fluorescent styryl dye FM1-43 (Li *et al.*, 2003; Wei *et al.*, 2013) at the zebrafish NMJ. Controls and C9-miR larvae with or without Calp and CAST treatments were exposed to FM1-43 and its uptake into NMJ presynaptic terminals were assessed. The presynaptic terminals were acutely depolarized with a

high [K+] Hank's Balanced Salt Solution (HBSS) (45 mM) to drive the synaptic vesicle release and recycling and label synaptic clusters with FM1-43. We observed marked reduction in fluorescent staining along terminal axon branches at individual synaptic varicosity boutons in C9-miR larvae compared to controls, indicating a slower exocytotic activity and synaptic vesicle cycle in C9-miR fish (Fig 4a,b). Treatment with Calp and CAST significantly improved the FM1-43 loading in presynaptic terminals in C9-miR larvae, we observed smaller puncta indicating a decrease of the FM1-43 loading (Fig 4a,b). Our data suggest that calpastatin modulates presynaptic vesicle release at the NMJ.

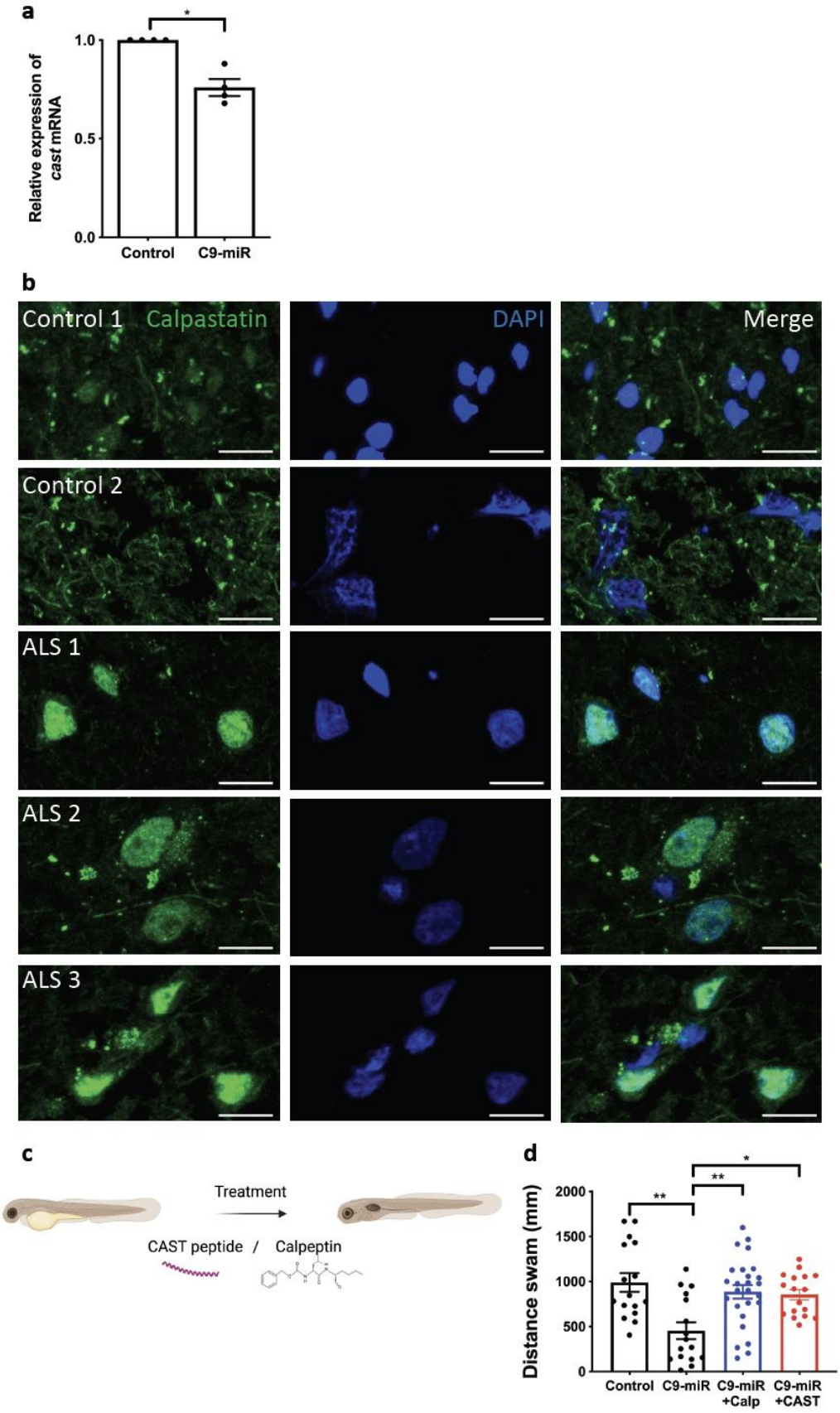

a
Relative expression of cast mRNA
1.0
0.5
0.0
Control
C9-miR
b
Control 1
Calpastatin
DAPI
Merge
Control 2
ALS 1
ALS 2
ALS 3
c
Treatment
CAST peptide / Calpeptin
d
Distance swam (mm)
2000
1500
1000
500
0
Control
C9-miR
C9-miR +Calp
C9-miR +CAST

Figure 3-1: Calpastatin dysregulation in ALS

a Bar graph shows the relative expression of the endogenous calpastatin gene. mRNA was normalized to elf1α mRNA level (N=4, p=0,0286). **b** Immunostaining of ALS patient (N=3) motor cortex tissue indicating a nuclear localization of calpastatin compared to a control with no neurological disorder (N=2). **c** Schematic representation of the treatments on C9-miR fish. C9-miR fish were exposed to calpastatin peptide labeled as CAST and calpeptin for four days starting at 2 dpf and all results were obtained at 6 dpf. **d** Treated C9-miR larvae displayed improved swimming behavior (CAST: N=3; n= 17; *p=0,0433) (Calp: N=3; n=26; **p=0,071, One way ANOVA). Data are presented as mean $\pm$ SEM. N represents the number of experimental repeats and n represents the number of fish.

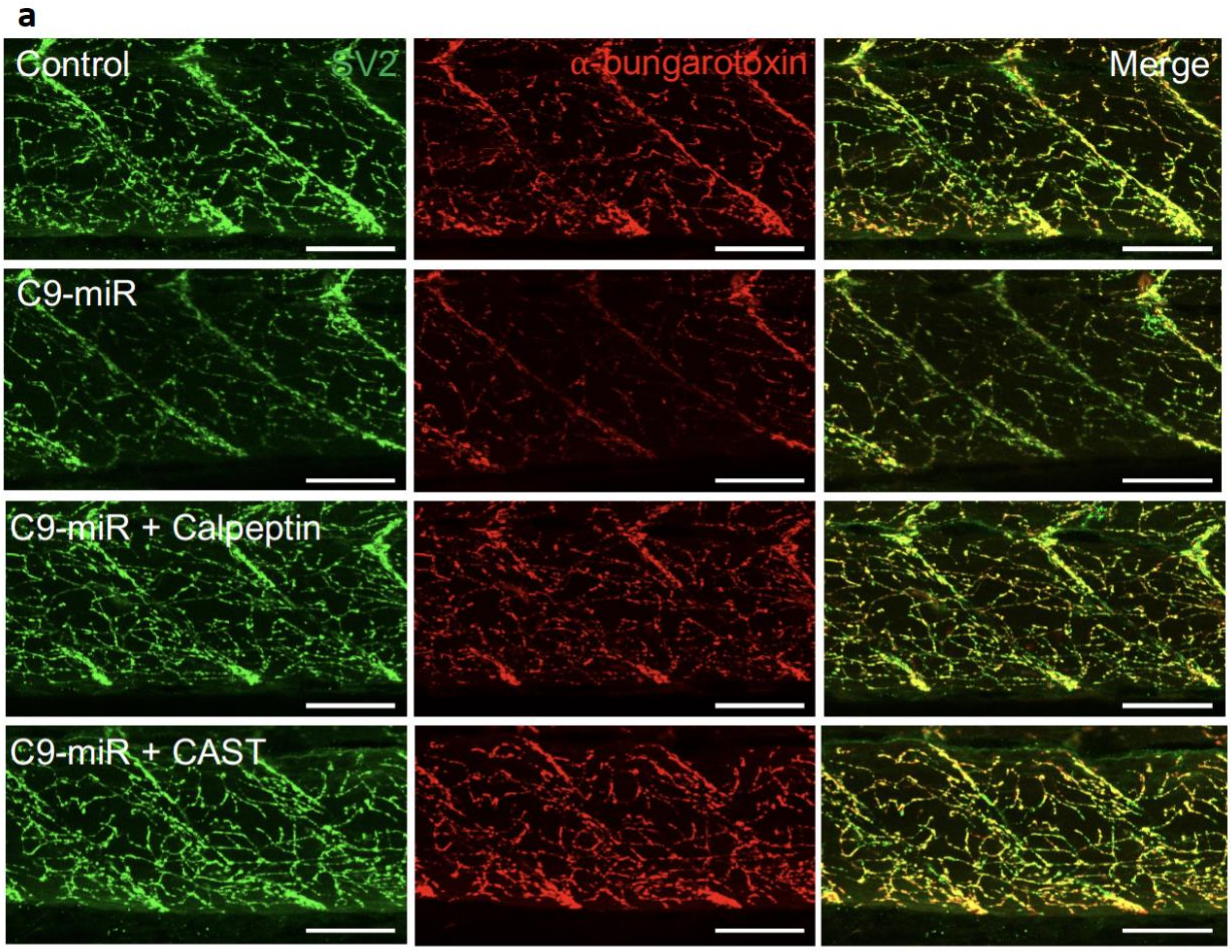

a
Control
SV2
α-bungarotoxin
Merge
C9-miR
C9-miR + Calpeptin
C9-miR + CAST
b
Mander's coefficient for colocalization of a-bungaro/SV2
0.4
0.3
0.2
0.1
0.0
**
**
*
Control
C9-miR
C9-miR +Calp
C9-miR +CAST

Figure 3-2: Rescue of the number of acetylcholine clusters at the neuromuscular junction (NMJ).

a Representative images of co-immunostaining of zebrafish neuromuscular junction with presynaptic (SV2; green) and postsynaptic (α-bungarotoxin; red) markers in 6 dpf zebrafish showing a rescue of the number of NMJ clusters in both treatments. Scale bar = 100μm. **b** Quantification of the colocalizing pre- and post-synaptic markers showing a significant increase of the NMJ number in both treated fish compared to C9-miR (Calp: n=7-8; **p=0,0014, One way ANOVA) (CAST: n=4-7; *p=0,0178, One way ANOVA).

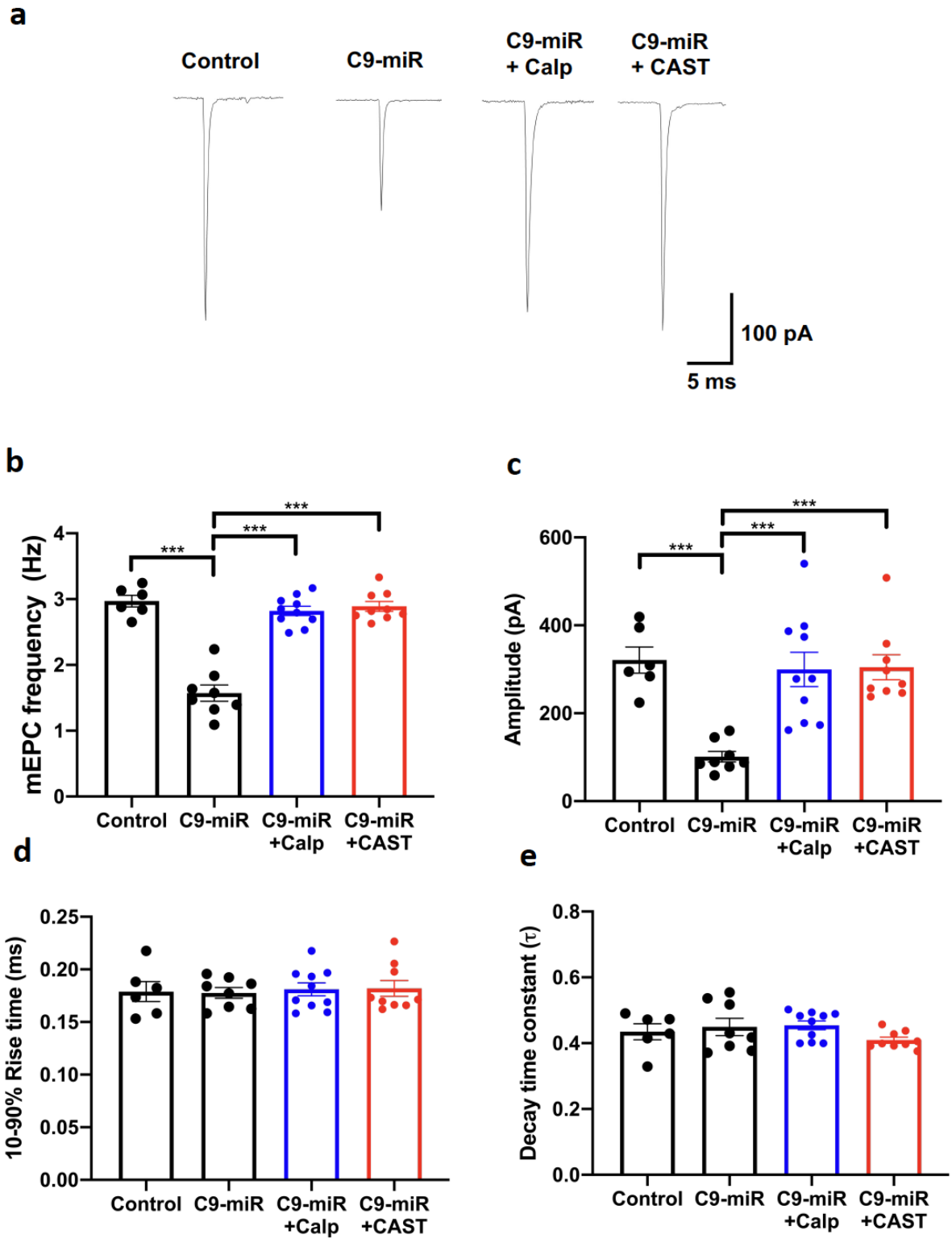
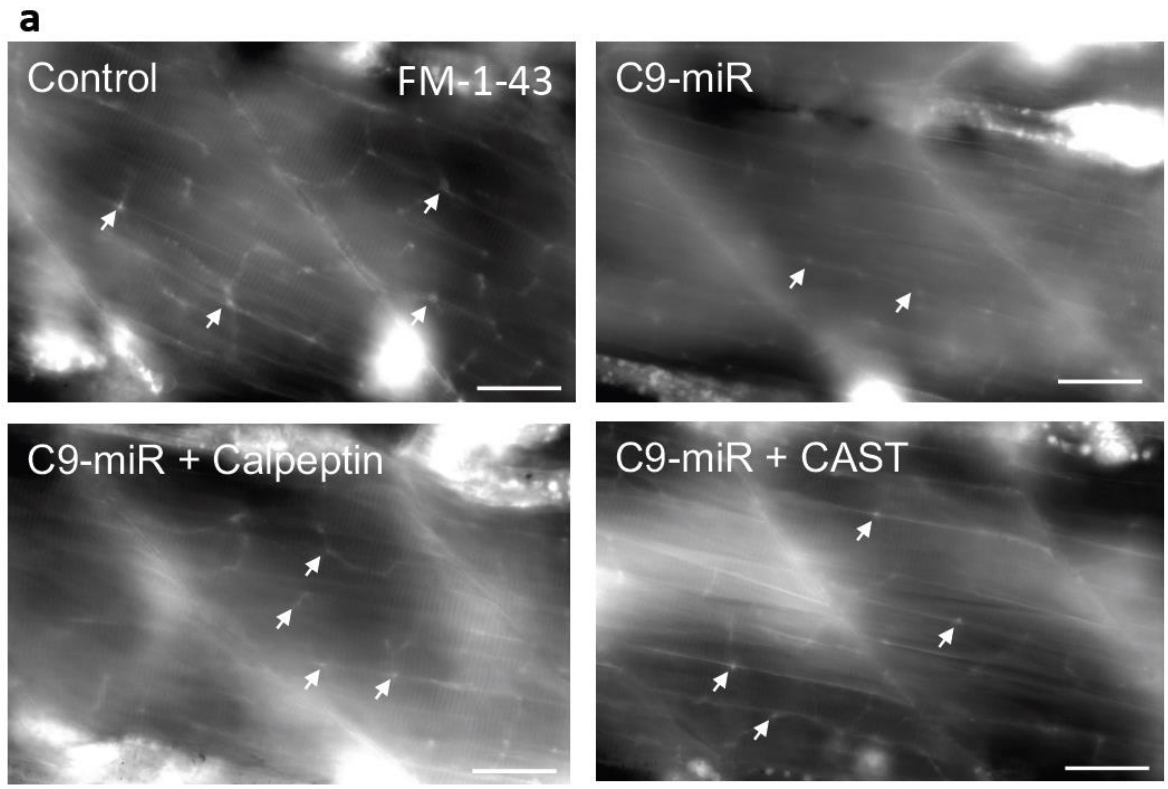

a
Control
C9-miR
C9-miR + Calp
C9-miR + CAST
100 pA
5 ms
b
mEPC frequency (Hz)
***
***
***
Control
C9-miR
C9-miR +Calp
C9-miR +CAST
c
Amplitude (pA)
***
***
***
Control
C9-miR
C9-miR +Calp
C9-miR +CAST
d
10-90% Rise time (ms)
Control
C9-miR
C9-miR +Calp
C9-miR +CAST
e
Decay time constant (τ)
Control
C9-miR
C9-miR +Calp
C9-miR +CAST

Figure 3-3: Treatments rescue miniature endplate currents (mEPCs) amplitude and frequency at the NMJ in C9-miR fish.

a Representative mEPCs. Fish treated with both compounds show a rescued frequency (**b**) (N=6-10; ***p<0,0001; One way ANOVA) and amplitude (**c**) (N=6-10; ***p<0,0005; One way ANOVA). Rise time (**d**) (N=6-10; p>0,05; One way ANOVA) and decay time (**e**) (N=6-10; p>0,05; One way ANOVA) were not found to be significantly different.


a
Control
FM-1-43
C9-miR
C9-miR + Calpeptin
C9-miR + CAST

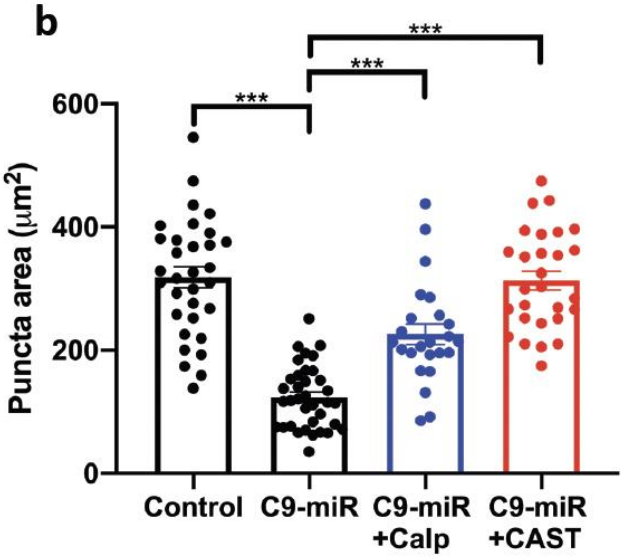

b
***
***
***
600
400
200
0
Puncta area (μm²)
Control
C9-miR
C9-miR +Calp
C9-miR +CAST

Figure 3-4: Rescue of the synaptic vesicle turnover.

a Representative images of the loading of FM1-43 dye at the NMJ in 6 dpf fish showing a significant rescue of the puncta area indicating a rescue of the synaptic vesicles turnover in treated fish. **b** Quantification of FM1-43 puncta area in control (N=8; n=32), C9-miR (N=8; n=36), C9-mir+Calp (N=6; n=24; ***p<0,001) and C9-miR+CAST (N=7; n=28; ***p<0,0001).

3.4 Discussion

Despite considerable advances in ALS research and therapeutic development, to date no curative treatment is available for ALS. Currently, the approved treatments have only mild beneficial effects on patients. Identification of novel therapeutic targets and compounds for ALS thus remains of utmost importance. Expression of calpastatin is markedly reduced in a zebrafish C9orf72 ALS loss of function model (C9-miR). We examined the effects of restoring Calpastatin function with two compounds, Calp and CAST, on neuromuscular dysfunction in C9-miR fish. We found that both Calp and CAST can improve impaired motor behavior in C9-miR fish. Importantly, these compounds restored NMJ structure and function in the C9-miR larvae. Our findings suggest that improving calpastatin function may be a potential therapeutic strategy for preserving NMJ integrity and motor function in ALS.

Calpastatin neuroprotective effects have been reported in several neurodegenerative diseases. For instance, in Huntington's disease, a treatment to prevent calpastatin degradation and stabilizing its function was found to reduce Huntington's disease neuropathology and ameliorate behavioral deficits mice models. This treatment, with the drug CHIR99201, also improved mitochondrial function and neuronal survival in Huntington's disease patient-derived neurons (Hu *et al.*, 2021). In hSOD1^{G93A} ALS mouse model, calpastatin improved hSOD1^{G93A} mice survival by 63 days and delayed the motoneuron axon death (Rao *et al.*, 2016). Here, we report pharmacologically mimicking calpastatin function with Calp and CAST in a zebrafish C9orf72 ALS model has beneficial effects. The effectiveness of compounds targeting the calpastatin system in SOD1 and C9orf72 ALS models strongly suggests new potential therapeutics for the treatment of ALS.

Synaptic dysfunction is a common feature of ALS pathology (Maselli *et al.*, 1993) and therapeutic strategies to preserve neuromuscular synapses may be impactful in treating ALS. Under physiological conditions, calpains have a role in neural development and synaptic transmission. During excitotoxicity with an overloading of calcium in the neurons, there is an induction of calpain activation leading to deleterious effects (Cheng *et al.*, 2018; Yildiz-Unal *et al.*, 2015). For example, calpain activation has been reported to trigger degradation of pre and post-synaptic components (Chan *et al.*, 1999). It has been shown that calpain can cleave synaptic proteins SNAP25 and SNAP23 impairing synaptic vesicle fusion and exocytosis (Ando *et al.*, 2005; Grumelli *et al.*, 2008; Lai *et al.*, 2003; Rutledge *et al.*, 2002; Wang *et al.*, 2017; Zimmerman *et al.*, 1999). Moreover, calpain activity upon intracellular Ca^{2+} accumulation results in cleavage of ALS proteins TDP-43, C9orf72, matrin3, VCP, profilin-1 and FUS (De Marco *et al.*, 2022). We demonstrated a

neuroprotective effect of the inhibition of the calpain with Calp or by restoring calpastatin function with CAST on motor behaviour and NMJ function in zebrafish C9-miR ALS model. Elucidating which precise synaptic components are cleaved upon alterations in the calpastatin/calpain system in ALS can provide more insights in synaptic dysfunction in the disease.

Restoring calpastatin function in zebrafish C9-miR fish with Calp and CAST preserved quantal synaptic transmission and synaptic vesicle cycle at NMJ. Dysfunctional NMJ transmission occurs at early stages in C9orf72 ALS (Devlin *et al.*, 2015; Sareen *et al.*, 2013). Our findings suggest that improving calpastatin function in ALS may prevent NMJ dysfunction and be an important therapeutic avenue to further investigate. Furthermore, reduction of the synaptic protein SV2a has reported in in cortical and motor neurons derived from C9orf72 patient induced pluripotent stem cell lines (Jensen *et al.*, 2020) and zebrafish C9-miR ALS model (Butti *et al.*, 2021). Expression of human calpastatin in a mouse model of SCS increases the expression of SV2a and significantly rescues impaired neuromuscular transmission at NMJ (Groshong *et al.*, 2007; Zhu *et al.*, 2013). It has been demonstrated that alterations in calpastatin/calpain system activate Cdk5 (Klinman *et al.*, 2015), a negative regulator of synaptic vesicle recycling and can repress presynaptic neurotransmission (Kim *et al.*, 2010; Shah *et al.*, 2014; Tan *et al.*, 2003). Overexpression of a Cdk5 inhibitory peptide in motor neurons of $SOD1^{G37R}$ ALS mice improves motor deficits, extends survival and delays neuroinflammation and pathology in brain and spinal cord of these mice (Bk *et al.*, 2019). Therefore, a thorough characterization of the calpastatin/calpain/Cdk5 cascade may provide new insights into synaptic communication and provides a wide array of potential targets for therapeutic intervention for ALS.

We demonstrated for the first time that in ALS patient motor cortex the localization of calpastatin is restricted within the nucleus. The inhibitory action of calpastatin on calpain activity depends on its cellular distribution to the cytoplasmic region. Our finding suggests that perhaps in ALS mechanisms involved in calpastatin cellular redistribution are altered. For instance, phosphorylation of calpastatin by protein kinase A plays a key role in its inhibitory ability to calpain (Adachi *et al.*, 1991; Du *et al.*, 2022). Interestingly, abnormal expression or activities of protein kinases have been reported in ALS (Krieger *et al.*, 2003). Further investigations are necessary to examine whether changes in protein kinases in ALS impact phosphorylation of calpastatin and subsequently its subcellular localization. Additionally, even though many studies support an overactivity of calpain in ALS upon altered calcium homeostasis, particularly with findings on cleavage of ALS-related proteins (De Marco *et al.*, 2022; Yamashita *et al.*, 2012), it will be

important to investigate changes in calpastatin and calpain expression and/or activity in ALS patients.

In summary, we have identified a decrease expression of calpastatin in a zebrafish C9or72 ALS model. Mimicking calpastatin function via calpain inhibition with Calp or addition of CAST, improved motor behavior and prevented NMJ defects in C9-miR zebrafish. Pharmacologically targeting the calpastatin/calpain system to preserve NMJ function in C9orf72 ALS may be a potential therapeutic approach to slow/halt ALS progression.

3.5 Methods

Zebrafish Husbandry

Adult zebrafish (*Danio rerio*) were maintained at 28°C at a light/dark cycle of 12/12 h in accordance with Westerfield zebrafish book. Embryos were raised at 28.5 °C and collected and staged as previously described. All the animal experiments were performed in compliance with the guidelines of the Canadian Council for Animal Care and received approval from the INRS-CNBE ethics committee.

Gene expression study

RNA was isolated from ~30 embryos using TriReagent® (Sigma) according to manufacturer's protocol. 1µg of RNA was used for cDNA synthesis using the SuperScript®Vilo™ kit (Invitrogen). RT-qPCR was run with SYBR Green Master Mix (Bioline) using the LightCycler® 96 (Roche). *ef1a* was used as the reference gene for normalization and following primers were used for *cast*:

Forward primer: 5'-ACAGACAAGTGCTCAAAAGGTG-3'; Reverse primer: 5' -GATCGTCAGCATCTGCACTG-3'.

Drug treatment

Zebrafish embryos at 48 hours post fertilization were placed in petri dishes and treated for 4 days in embryo medium (E3). Calpeptin was used at 10µM, stock was reconstituted in DMSO. Calpastatin peptide was purchased from Sigma-Aldrich (SCP0063) and reconstituted in distilled water to be used at 1µM. Both drugs and water were changed every 48 hours.

Behavioral assay

Larvae (6 dpf) were separated into single wells of a 96-well plate containing 200µl of E3 media and habituated in the the Daniovision® recording chamber (Noldus) for 1 hour before start of experiment. Larval locomotor activity was monitored over light-dark cycles using the Daniovision® apparatus. Analysis was performed using the Ethovision XT12 software (Noldus) to quantify the total swimming distance in given hours and the locomotor activity per second.

NMJ morphology in larval zebrafish

Immunohistochemical analyses were performed on 6 dpf zebrafish to visualize NMJ pre- and postsynaptic structures. Briefly, animals were fixed in 4% paraformaldehyde overnight at 4°C. After fixation, the larvae were rinsed several times (1 hour) with PBS-Tween and then incubated in PBS containing 1 mg/ml collagenase for 180 minutes to remove skin. The collagenase was washed off with PBS-Tween (1 hour), and the larvae were incubated in blocking solution (2% NGS, 1% BSA, 1% DMSO, 1% Triton-X in PBS (PBST)) containing 10 mg/ml Tetramethylrhodamine conjugated α-bungarotoxin (Thermofisher T1175) for 30 minutes. The larvae were rinsed several times with PBST (30 minutes) and then incubated in freshly prepared block solution containing primary antibody Sv2a (1:200, Developmental Studies Hybridoma Bank) overnight at 4°C. Following this, larvae were incubated in block solution containing a secondary antibody (Alexa fluor 488, 1:1,000, cat# A-21042, Invitrogen) for overnight at 4°C. The following day the larvae were washed several times with PBST and mounted on a glass slide in 70% glycerol. Z-stack images were taken using a Zeiss LSM780 confocal microscope (Carl Zeiss, Germany). The images were then processed with ZEN software (Carl Zeiss). Co-localization analysis of pre- and post-synaptic structures was performed using the JACOP program from ImageJ (NIH).

FM1-43 staining

Zebrafish larvae (6dpf) were first anesthetized in Evan's solution (134 mM NaCl, 2.9 mM KCl, 2.1 mM $CaCl_2$, 1.2 $MgCl_2$, 10 mM HEPES, 10 mM glucose) containing 0.02% tricaine (Sigma Aldrich). The larvae were then pinned to a Sylgard coated dish both at the head and extreme tail end using electrolytically sharpened tungsten needles. The skin was then carefully peeled away to expose the muscles and to permit access to FM1-43 (Molecular Probes). The fish were treated with Evan's solution containing 10 µM of FM1-43 to allow preloading penetration of the dye molecules.

After 10 min, the fish were transferred to a high potassium Hank Buffer Salt Solution (HBSS) (97 mM NaCl, 45 mM KCl, 1 mM $MgSO_4$, 5 mM HEPES, 5 mM $CaCl_2$) containing 10 μM of FM1-43 for 5 minutes. The fish were then tranferred in Evan's solution with 10 μM of FM1-43 finished for an additional 3 minutes, after which loading was complete. The fish were then washed with a low calcium Evan's solution (0.5 mM $CaCl_2$) three times for 5 minutes to minimize spontaneous release of loaded synaptic vesicles. The fish were imaged for FM1-43 staining using a 40X Examiner A1 microscope (Zeiss). Blind measurements of FM1-43 staining at NMJs in wild-type control and C9-mIR fish were performed using ImageJ (NIH).

Electrophysiology recordings

Whole-cell patch clamp recordings were taken from muscle cells of 6 dpf larvae. The preparation was bathed in an extracellular solution (134 mM NaCl, 2.9 mM KCl, 1.2 mM MgCl, 10 mM HEPES, 10 mM glucose, pH 7.8) containing 1 μM of tetrodotoxin (TTX; Tocris, UK) in order to block action potentials during mEPC recordings. Patch clamp electrodes (2-4 MΩ) were filled with an intracellular solution (130 mM CsCl, 8 mM NaCl, 2 mM $CaCl_2$, 10 mM HEPES, 10 mM EGTA, 4 Mg-ATP, 0.4 Li-GTP, pH 7.4). Miniature endplate currents from white muscle fibers were recorded in the whole-cell configuration with an Axopatch 200B amplifier (Molecular Devices) at a holding potential of -60 mV, low-pass filtered at 5 kHz and digitized at 50 kHz. Series resistance was compensated by at least 85% using the amplifier's compensation circuitry. Synaptic currents were acquired using the pCLAMP10 software (Molecular Devices).

Analysis of miniature endplate currents

Miniature endplate currents (mEPCs) were analyzed using the AxoGraph X software. The mEPC recordings were examined by the software, and synaptic events were detected using a template function. Overlapping or misshapen events were removed and the remaining events were averaged and the properties (amplitudes, decay time constants, frequencies) of the averaged trace were measured. Events with slow rise times and low amplitudes were excluded from the analysis, therefore, only fast rise time events were included in our analysis since these events originated from the cells that were patch clamped rather than from nearby, electrically-coupled muscles. Single decay time constants were fit over the initial (fast) decay portion and over the distal (slow) portion of the decay. For each *n*, currents were recorded from a single white muscle fiber from a single larva.

Post-mortem tissue staining

Control (N=2) and ALS (N=3; genetic cause undetermined) samples from patient motor cortex were obtained from the CHUM biobank. Samples had previously been embedded in OCT. Slides with the sections were thawed followed by two successive baths of PBS-Tween20 (PBS-T) and processed for epitope retrieval in citrate solution (10mM, pH 6) for 20 minutes at 68°C. Sections were then rinsed several times in PBS-T and then incubated in block solution (1%NGS in PBS-T) with primary antibody for Calpastatin (1:200, Invitrogen 1F7E3D10) overnight at 4°C. After washing, sections were incubated in block solution containing secondary antibody (Alexa fluor 488, 1:1000, cat# A-21042, Invitrogen) for 2 hours followed by rinsing and a 15 minutes incubation in Hoechst solution (1:10 000, Invitrogen). Sections were finally rinsed several times in PBS-T and mount in Fluoromount media. Z-stack images were taken using a Zeiss LSM780 confocal microscope (Carl Zeiss, Germany).

Statistical analysis

All zebrafish experiments were performed on at least three replicates (N) and each consisted of a sample size (n) of 5-30 fish. Data are presented as Mean±SEM. Significance was determined using either Student's t-test or One-way ANOVA followed by multiple comparisons test. A Tukey post-hoc multiple comparisons test was used for normally distributed and equal variance data. Kruskal-Walllis ANOVA and Dunn's method of comparison were used for non-normal distributions. All graphs were plotted using the Graphpad PRISM software. Significance is indicated as * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

4 DISCUSSION, PERPECTIVES AND CONCLUSION

Since the discovery of the gene *C9orf72* as a causative gene in ALS in 2011, many hypotheses have emerged and three mechanisms proposed to explain the C9 pathology have been presented as mutually exclusive for some time. Indeed, many studies have been reporting that only toxic gain of function (GOF) mechanisms involving RNA foci formation and RAN translation leading to dipeptide production were responsible for the disease. Whereas some other studies would defend the loss of function (LOF) mechanism. Multitude of models have been generated to unravel the C9 pathology and find treatments. Both toxic GOF mechanisms have been widely studied and led to potential treatments to decrease their toxic effects. Antisense oligonucleotides (ASOs) therapies, for example, have been tested in clinical trial conducted by Biogen although it resulted in a failure of efficiency in patients. Immunotherapy has also been tested in mice, using antibodies to target poly-GA dipeptide and bring them to degradation which have been showing a failure of efficiency as well (Jambeau *et al*, 2022). Both failures suggest that targeting only GOF mechanisms may not be enough and that LOF mechanism may have a non negligeable role in the C9 pathology. *C9orf72* HRE involves many dysfunctions which makes it very difficult to find efficient treatments. Understanding the endogenous role of the gene and how its loss of expression contributes to the disease was the purpose of the project. Thus, we have generated a new C9-LOF model in zebrafish using micro-RNAs. We have been knocking down the gene to mimic the decrease found in patient post-mortem tissues (Belzil *et al.*, 2013) (Fratta *et al.*, 2013) (van Blitterswijk *et al.*, 2015; Waite *et al.*, 2014) (Rizzu *et al.*, 2016) and assessed the synaptic phenotypes upon the LOF.

In this thesis, we have (1) generated and characterized a new knockdown model for *C9orf72* presenting the main ALS featured including motor behaviour, loss of neuromuscular junction (NMJ) and the TDP-43 pathology, (2) identified a role for C9orf72 at the synaptic junction and more precisely at the NMJ, indeed, our mutant showed reduced expression of SV2, Rab3a and an impairment of the synaptic vesicles recycling. Lastly (3), we identified calpastatin as neuroprotective in our model and restoring the balance between calpain and calpastatin could rescue some ALS related phenotypes.

4.1 Generation of a loss of function model

Several LOF models for *C9orf72* did not recapitulate motor defects and motoneuron loss as observed in ALS. Indeed, mice *C9orf72* LOF models present more inflammatory response to the

mutation and fail to develop any ALS related phenotypes (Atanasio *et al.*, 2016; Burberry *et al.*, 2016; Jiang *et al.*, 2016; Koppers *et al.*, 2015; Lagier-Tourenne *et al.*, 2013; O'Rourke *et al.*, 2016; Sudria-Lopez *et al.*, 2016; Sullivan *et al.*, 2016; Ugolino *et al.*, 2016). Mice have been widely used to model GOF mechanisms and reported ALS like phenotypes (Herranz-Martin *et al.*, 2017; Liu *et al.*, 2016b; Pattamatta *et al.*, 2021; Riemslagh *et al.*, 2021a). This implicated the theory claiming that LOF is not involved in ALS pathogenesis. Although, in 2019, a mouse model combining LOF and GOF showed that the mechanisms were acting in a symbiotic way (Shao *et al.*, 2019). In drosophila, GOF mechanisms were modelized using UAS/Gal4 system or stable repeats RNA expression. These models showed several ALS related phenotypes including neurodegeneration (Freibaum *et al.*, 2015; Mizielinska *et al.*, 2014; Moens *et al.*, 2018; Xu *et al.*, 2013). Zebrafish GOF models have also been generated and both RNA and DPR injection have been showing toxic effects (Lee *et al.*, 2013; Ohki *et al.*, 2017; Shaw *et al.*, 2018; Swaminathan *et al.*, 2018; Swinnen *et al.*, 2018). Transient and stable expression of the GGGGCC repeats triggered motoneuron impairment including axonopathy and loss of NMJ, reduced motor functions, as well as muscle atrophy and early mortality. Nevertheless, the level of *C9orf72* mRNA has not been investigated in these models, which would give insights about the role of the RNA repeats and the DPR on *C9orf72* expression. Moreover, GOF models have been created by overexpressing GGGGCC RNA and/or dipeptides which may not reflect the clinical condition found in patients which can bias the observed phenotypes. Lastly, *C9orf72* fish ortholog does not present the GGGGCC repetitions in its genome so all GOF models were generated by injecting exogenous construct, this may explain their toxicity. LOF in iPS cells have been showing several dysfunctional mechanisms in relevant cell types including patient derived motoneurons although it remains a cellular model that is not able to mimic the disease at a whole organism level (Shi *et al.*, 2018). *C.elegans* knockout model for *C9orf72* ortholog featured motility defects and paralysis and in the same way, the first knockdown model for *C9orf72* in zebrafish showed similar results including axonopathy and motor behaviour impairments (Ciura *et al.*, 2013; Therrien *et al.*, 2013). Yet, having a stable model to study the late stages of the disease was a key of our project. Indeed, morpholinos used in the zebrafish have an off-target effect that can lead to altered results and are also, only efficient during a transitory moment preventing from studying late stages of the disease. Using the Tol2 system allows a stable integration of our micro-RNAs in the zebrafish genome, in addition, the design of the four synthetic micro-RNAs allowed to determine the downregulation of the gene (Giacomotto *et al.*, 2015). Indeed, we targeted a down regulation of *C9orf72* that would reflect what the decrease found in patient cells. In this project, we have created a new mutant zebrafish line that recapitulates ALS main phenotypes thanks to a decrease of *C9orf72* ortholog

expression. This model shows that *C9orf72* LOF engenders motor defects with a decrease motor activity, a higher and earlier mortality, muscle and motoneuron atrophy as well as a loss of NMJ number. Together, these data strongly suggest that *C9orf72* LOF is involved in ALS pathology and that it is enough to trigger disease like phenotypes. We have also been testing the effect of repeats expression in our model and observed that a 91(GGGGCC) repeat would have a high toxicity. This toxicity is also observed in wild type fish (Swinnen *et al.*, 2018) although, our mutant model shows a higher sensitivity to these repeats indicating that the three mechanisms proposed for the C9orf72 pathology may work together and have a synergic effect (Pal *et al.*, 2021). Generating a model expressing the GGGCC in the first intronic region of the zebrafish ortholog of *C9orf72* and studying the mRNA and all ALS related phenotypes may be the most accurate model.

4.2 C9orf72 role at the synaptic junction

4.2.1 C9orf72 role and loss of function

When C9orf72 has been found to be associated with ALS in 2011, its role was unknown (DeJesus-Hernandez *et al.*, 2011) (Renton *et al.*, 2011). With bioinformatic studies, the differentially expressed in normal and neoplasia (DENN) domain has been identified giving a clue about a GDP/GTP exchanging factor (GEF) for Rabs-GTPases (Levine *et al.*, 2013). Rabs-GTPases are involved in regulation of membrane trafficking so C9orf72 could be a regulator of this traffic having a role in autophagy and/or at the synapses. Additionally, several Rabs are compartment specific like Rab3 which was found enriched at the synapse in flies (Kiral *et al.*, 2018). Rab3 in involved in the docking of synaptic vesicles to allow their exocytosis in the synaptic cleft (Geppert *et al.*, 1997).

C9orf72 has been localized in the synaptic compartment in different studies. For instance, in developing mice, C9orf72 has been found in the synaptosome fraction while in adult mice brain, C9orf72 was found in the synaptic fraction (Atkinson *et al.*, 2015). More precisely, C9orf72 was found in the pre-synaptic compartment and interacting with Rab3 protein in mouse brain (Frick *et al.*, 2018). Even more recently, C9orf72 expression was again identified in both pre- and post-synaptic compartments and interacting with Rab39b at the post-synaptic level (Xiao *et al.*, 2019). All of these data point towards a role for C9orf72 at the synapse and investigating the LOF would unravel some of its mechanistic implications at the synapse.

Additionally, in a very recent study, C9orf72 is reported to interact with synapsin (Bauer *et al.*, 2022). This study reports also that a *C9orf72* LOF reduces the number of excitatory synapses *in vitro*, in neuronal cultures and *in vivo*, in hippocampus mouse brain. *C9orf72*-KO mice in the study also showed reduced number of synaptic vesicles at excitatory synapses. Besides, this study mentions that synapsin is an effector of Rab3a which suggests that C9orf72 role is at a turning point in synaptic vesicle traffic at the synaptic cleft. Altogether, this new study indicates that C9orf72 plays a great role in neurotransmission and more precisely at excitatory synapses.

In our study, we have found that the *C9orf72* mutant fish had significantly less Rab3a positive puncta at the NMJ, suggesting that C9orf72 is involved in Rab3a regulation of expression and/or localization, explaining the observed decrease. Since C9orf72 has been showed to be a GEF for Rab8 and Rab39 (Corbier *et al.*, 2017), we can suppose that C9orf72 is involved in the regulation of other Rabs. Also, Xiao and colleagues reported that C9orf72 regulation of Rab39b in the synaptic compartment has a role in glutamatergic receptor regulation which indicated that C9orf72 has a key role at the synaptic cleft in ALS (Xiao *et al.*, 2019). Besides, we have observed a loss of recycling of the synaptic vesicles with the FM1-43 staining. This suggests that C9orf72 could have a regulatory role for Rabs involved in synaptic vesicle recycling such as Rab27b. Indeed, murine neurons mutant for Rab27b have been reported to have a strongly impaired synaptic vesicle recycling (Pavlos *et al.*, 2010). Rab5a could also be investigated since it has been found to decrease synaptic vesicle recycling when mutant in drosophila and in rats, hippocampal neurons overexpressing Rab5a showed a decrease recycling as well (Shimizu *et al.*, 2003; Star *et al.*, 2005). So Rab5a seems to be strongly implicated in synaptic vesicle recycling. All these dysregulated mechanisms lead to a loss of the NMJ observed with the SV2 and α-bungarotoxin co-staining. Indeed, the loss of exocytosis at the synaptic cleft has been linked to reduced frequency and amplitude of the miniature end plate currents (mEPCs) at the NMJ in our mutant and the inefficacy in synaptic transmission leading to a loss of the NMJ. mEPCs being spontaneous signals sent from the motoneuron to the muscle not provoking an action potential but to keep the NMJ stimulated and healthy. The decrease of frequency reflects a decrease of the frequency of synaptic vesicle release and the decrease of amplitude reflects a decrease of quantal content (Armstrong *et al.*, 2013a). Altogether, our LOF model suggests that C9orf72 is a key regulator of the synaptic transmission at the NMJ, by regulating pre-synaptic components. Its downregulation triggers a dysregulation of the transmission leading to a defective NMJ and its loss. This could explain how knockdown therapy targeting the GGGGCC repeat expansion by ASOs can fail to protect the NMJ.

4.2.2 Link with SV2a

The substantial loss of NMJ contacts and the decreased frequency and amplitude of mEPCs made us look deeper into the potential mechanisms that could be dysregulated. Our mass spectrometry analysis pointed out that SV2a was the most downregulated protein in our mutant. We confirmed the loss of SV2a using immunostaining at NMJs. Interestingly, SV2a downregulation has also been reported in a C9orf72 patient-derived iPS cells model which also displayed synaptic dysfunction and a decrease of both mRNA and protein levels of SV2a (Jensen *et al.*, 2020). Using a mouse model expressing 149 poly-GA repeats, it was also reported that neurons expressing the repeats showed a SV2a loss of expression. These neurons displayed a dysfunctional calcium influx with increased intracellular calcium concentration which led to a lack of synaptic vesicle release. The synaptic dysfunction observed in these neurons was rescued by an upregulation of SV2a levels indicating a neuroprotective effect (Jensen *et al.*, 2020). SV2a is found in the active zone at the synaptic junction and is presumably involved in several processes that lead to the synaptic transmission. While it has been extensively studied, the role of SV2 remains unclear. Since 1992, SV2a has been proposed to be involved in neurotransmitter loading into synaptic vesicles and has been related to calcium signalling mediator to regulate synaptic vesicle fusion (Feany *et al.*, 1992). A knockout mouse model for SV2a showed decreased amount of synaptic vesicles pool with a diminished release probability and reduction in spontaneous events (Crowder *et al.*, 1999; Custer *et al.*, 2006). SV2a has also been proposed to have a role in maintaining and orienting the releasable pool of synaptic vesicles and in the coordination of the neurotransmitter release, more particularly in the peripheral nervous system (Vogl *et al.*, 2015). This data suggests that SV2a loss of expression is involved is the decreased frequency of the mEPCs at the NMJ. Moreover, we have found that SV2a interacts with C9orf72 using a co-immunoprecipitation technique indicating that C9orf72 most likely is involved in its regulation. Altogether, the findings suggest that SV2a could have a role in neurotransmitter loading in the synaptic vesicle and in the synaptic release, and these processes seem to be regulated by C9orf72. This correlates with the observation we made at the NJM with decreased frequency and amplitude of mEPCs reflecting a lack of vesicle release and quantal content evoked in the previous part.

Lastly, SV2a is the target of an antiepileptic drug, Levetiracetam (Lynch *et al.*, 2004). Similarly, Ezogabine, which is another antiepileptic drug that was tested in clinical trial for ALS (Wainger *et al.*, 2021), Levetiracetam could be tested on our model to assess a potential therapeutic effect.

Indeed, the very specific anatomical distribution of SV2a makes it a highly interesting target to avoid off target effect and side effects.

4.3 C9orf72 loss of function and TDP-43 aggregation

TDP-43 aggregation in C9orf72 pathology is a conundrum many teams have been investigating. Both GOF and LOF have been found to be causative in this process although this pathology has been difficult to reproduce in both GOF and LOF models. In our *C9orf72* LOF model, we have observed a perinuclear TDP-43 aggregation. The mechanism triggering the mislocalization in our model remains unclear, but it could be explained by a dysregulation of the nucleocytoplasmic transport (NCT). Indeed, C9ORF72 patient tissues showed a cytoplasmic localization of importin-β1 and C9orf72 derived iPS cells showed a dysregulation of seven nucleoporins including nup107 (Coyne *et al.*, 2020; Kinoshita *et al.*, 2009; Saberi *et al.*, 2018). Recently, toxic GOF mechanism as the three dipeptides expression, poly-GA, poly-GR and poly-PR, have been identified as disruptive for the NCT leading to a mislocalization of TDP-43 in HeLa cell (Ryan *et al.*, 2022). It had already been showed in 2020 that a 200 poly-GR expression promotes this aggregation and TDP-43 recruitment to stress granules (Cook *et al.*, 2020). Also, mice expressing 149 GGGGCC repetitions treated with ASOs against the repeats showed an improvement of the poly-GR and TDP-43 aggregation phenotype. In opposition to the previous studies, it has been reported that a mouse model expressing poly-GR would not recapitulate the TDP-43 pathology (Verdone *et al.*, 2022). Another study using embryonic cortical mouse neurons treated with short hairpin RNA (shRNA) to reduce *C9orf72* expression could trigger TDP-43 aggregation indicating that LOF mechanism may also be involved in this pathological mechanism (Sellier *et al.*, 2016). Although, it was also reported that hypothalamic neurons did not show the same phenotype, suggesting that this whole TDP-43 aggregation mechanism would be time and/or cell dependent. Altogether, these results seem to show that both GOF and LOF could be involved in TDP-43 mislocalization. Finally, iPS derived neurons, astrocytes and mice neurons presenting TDP-43 aggregation showed that the aggregates can be cleared through the autophagy pathway (Barmada *et al.*, 2014; Wang *et al.*, 2013). So, a LOF of *C9orf72* could explain how TDP-43 accumulates in the cell cytoplasm. Indeed, an overexpression of *C9orf72* could reduce mutant TDP-43 aggregation, this latter mechanism could be explained by the lack of autophagy related components regulated by C9orf72 like ATG13 for example (Nguyen *et al.*, 2019; Shao *et al.*, 2020).

4.4 Calpastatin neuroprotective effect at the NMJ

Calpain is a calcium-dependent cysteine protease and has been previously reported as overactivated in pathological conditions like neuromuscular diseases (Spencer *et al.*, 2002; Stifanese *et al.*, 2014; Wootz *et al.*, 2006). Besides, it has been linked to cleavage of both pre- and post-synaptic components at the synaptic cleft (Chan *et al.*, 1999). Inhibition of calpain by the calpastatin has a neuroprotective effect, which has been studied in neurodegenerative diseases like Alzheimer's, Parkinson's, Huntington's diseases and FTD (Diepenbroek *et al.*, 2014; Hu *et al.*, 2021; Rao *et al.*, 2016; Rao *et al.*, 2014; Rao *et al.*, 2008; Tradewell *et al.*, 2010). Also, treatment targeting this pathway has been showing promising results in Huntington's disease mouse model (Hu *et al.*, 2021). Calpain has been reported as involved in TDP-43 cleavage to a pro-aggregation form (Yamashita *et al.*, 2012). Here, our model presents a decreased expression of the calpastatin. Perhaps TDP-43 aggregation in our model could be a consequence of calpastatin loss of expression. Calpain has also been linked to neurodegeneration through the activation of Cdk5 (Klinman *et al.*, 2015). Cdk5 is a negative regulator of synaptic vesicle recycling and can repress presynaptic neurotransmission (Kim *et al.*, 2010; Shah *et al.*, 2014; Tan *et al.*, 2003). Calpain can also cleave proteins involved in synaptic vesicle fusion and exocytosis SNAP25 and SNAP23 leading to a lack of efficacity (Ando *et al.*, 2005; Grumelli *et al.*, 2008; Lai *et al.*, 2003; Rutledge *et al.*, 2002; Wang *et al.*, 2017; Zimmerman *et al.*, 1999). Therefore, calpastatin loss of expression in our model could be involved in the decrease of expression of SV2a and the reduced synaptic transmission at the NMJ. We showed that by treating our fish with calpeptin, a calpain inhibitor, or with a calpastatin peptide encoding the 27 amino acids of the active site, the synaptic transmission, synaptic vesicle turnover defect, the NMJ integrity and the motor activity were rescued in our mutant. In addition, calpeptin compound and peptide treatment did not show any toxicity in our fish leading to think that this is a potential therapeutic pathway to explore further.

Lastly, in human patient's brain, we have found that calpastatin was more localized in the nucleus, suggesting a lack of activation. Indeed, phosphorylation of calpastatin by protein kinase A is involved in calpain inhibition (Adachi *et al.*, 1991; Du *et al.*, 2022) and abnormal expression or activities of protein kinases have been reported in ALS (Krieger *et al.*, 2003). In normal condition, calpain activation triggers its own inhibition by activating calpastatin. Although, our model shows that C9-LOF engenders a calpastatin LOF which can lead to toxic pre- and post-synaptic components cleavage by calpain. Taken together, the results indicate that C9orf72 could be a regulator of the calpastatin/calpain pathway.

Another indirect mechanism could be that *C9orf72* LOF leads to excitotoxicity, leading to calpain activation by glutamate stimulated calcium rise. This excitotoxic mechanism reported in patients combined with calpastatin mislocalization in the nucleus indicating the lack of activation suggests a strong dysregulation of the pathway in ALS.

4.5 Perspectives

4.5.1 Studying both *C9orf72* GOF and LOF in a stable line

Generating a line with a ubiquitous LOF of *C9orf72* has been helping to identify defects at the NMJ. As a future direction, it is important to generate a model with both LOF and GOF expressed in a stable manner, such as GGGGCC repeats in the first intron of the zebrafish ortholog for *C9orf72*, to have a model as close as it can to the human mutation. Indeed, in our model, we have been injecting mRNA containing the GGGGCC repeats and we have observed a high sensitivity to these repeats in our LOF mutant compared to wild type fish. This finding has been confirmed in a mouse model showing a higher sensitivity to the repeats toxicity when *C9orf72* is knock down using antisense oligonucleotides (Zhu *et al.*, 2020). Studies have been linking GOF toxicity to synaptic dysfunction as well, so seeing the synergistic mechanisms in action could unravel some new insights in the synaptic defects seen in patients (Jensen *et al.*, 2020) (Freibaum *et al.*, 2015; Herranz-Martin *et al.*, 2017; Xu *et al.*, 2018; Zhang *et al.*, 2015). Additionally, studying both GOF and LOF will provide more insight on TDP-43 aggregation.

4.5.2 Elucidating the motoneuron loss and synaptic defects

Here we have showed that *C9orf72* LOF leads to an alteration of synaptic vesicle release and recycling leading to a loss of NMJ. Although we have not yet investigated the hyperexcitability in our model. Electrophysiology studies will help to know if our model shows hyperexcitability at an early stage. Indeed, cortical hyperexcitability being a major feature of the disease (Vucic *et al.*, 2014), investigating both brain and spinal neurons in our fish would bring insight into this mechanism. Also, patient derived iMNs have been reported to display a hyperexcitability phenotype, this would confirm the role of C9orf72 LOF in this process (Burley *et al.*, 2022). All of our ALS related phenotypes have been identified at 6 days post fertilization (dpf), which means, that the mechanisms leading to these phenotypes could be investigated earlier to identify where the pathological mechanism starts. Doing pair recording between the interneuron and the motoneuron will give insights about the inputs the motoneuron receive and explain how the

mEPCs are affected. Indeed, a study has showed that patient derived neurons were presenting a hyperexcitability which was followed by a loss of synaptic activity (Devlin *et al.*, 2015).

Using the advantages of our transparent zebrafish model, we could also perform an *in vivo* analysis of the axonal trafficking. Indeed, C9orf72 pathology leads to axonal transport defect in patients (Gendron *et al.*, 2017; Mizusawa *et al.*, 1989; Munoz *et al.*, 1988). By injecting a fluorescent kinesin or dynein at the one cell stage in our model, we could assess the axonal transport by live imaging with a two-photon microscope for example. This would indicate if C9orf72 LOF is involved in the axonal transport defects observed in ALS.

Besides, investigating *in vitro* the regulation mechanism between C9orf72 and SV2, whether they act as a complex or if this is a transitory activation would be to explore. Using techniques such as Bioluminescence energy transfer or BioID experiments for example, it would be possible to know if the interaction is transitory. Also, bioinformatic programming could also predict the possible interaction sites between C9orf72 and SV2, indeed, protein conformation platform such as DeepMind is currently developing algorithm to predict protein conformation, so the next step could be to predict protein-protein interaction site.

Lastly, C9orf72 could be investigated as a Rab5a regulator, since Rab5a in an activator of Phospatidylinositol-3 kinase (PI3K) (Shi *et al.*, 2019). Recently, Phospatidylinositol-3 phosphate (PI3P) decrease, synthetized by PI3K, has been linked to Cdk5 activation through calpain cleavage leading to a lack of synaptic vesicle recycling and neurotransmitter release (Liu *et al.*, 2022). PI3P and Cdk5 levels could also be assessed in our model and be targeted using pharmacological molecules as potential therapeutic targets.

4.5.3 Investigating FTD related phenotypes

As mentioned at the very beginning of the manuscript, *C9orf72* is the most common genetic cause of ALS and it is in the middle of the spectrum going from ALS to fronto-temporal dementia (FTD). While zebrafish does not have a cortex thus, no frontal or temporal lobes, pallilum and subpallium regions of the zebrafish brain are involved in cognitive functions. Studying brain functions and synaptic transmission using electrophysiological recordings in these regions could provide more insight in C9orf72 role in the brain. Our model presents several advantages to study brain morphology as well using immunofluorescent staining for neuronal markers, it can help get *in vivo* insights on FTD. Also, it has recently been reported that 14% of FTD patients had a smaller hypothalamus volume (Shapiro *et al.*, 2022) so this could be investigated in *C9orf72* LOF fish.

TDP-43 aggregation in the mutant fish brain could be investigated as well as other proteinopathies found in FTD such as Tau or FUS aggregation.

4.5.4 Calpastatin defects in other ALS models

In this project, we mainly focused our research on C9orf72 pathology in ALS. Patient mislocalization of calpastatin suggests an alteration of the calpain/calpastatin system. This is something to be investigated in other ALS models and confirmed to know if a global therapeutic approach can be developed. In the upcoming months, we will investigate the level of calpastatin in both C9orf72 and sporadic ALS patient's derived motoneurons and its localization. We will also treat these motoneurons using calpeptin and calpastatin peptide to assess their effect on cell survival measuring the level of ATP. Level of ATP released by cell being directly proportional to the number of cells in culture, this will reflect cell survival. These experiments will bring insights on calpastatin neuroprotective effect in a cell relevant model. These motoneurons could be used as well to measure electrophysiological properties using path clamp technique. To go further, these treatments could be tested in higher animal models to assess their efficacy and toxicity in mammals.

4.6 Conclusion

In conclusion, during this project, we have generated and characterized a new model to study *C9orf72* LOF as a model for ALS. Upon *C9orf72* LOF, this model showed a decrease survival and motor function with a loss of NMJ. Our model can be used as a new tool to make *in vivo* analysis as well as drug treatment screenings to narrow down the number of drugs to test on higher organisms, such as mice, and thus, reducing the number of animals needed.

Our model brought a new insight into *C9orf72* LOF which has been described as non-pathological in ALS for some time, here, we show that upon a LOF, our model resumes ALS main features. Also, by injecting repeats in our model, we were able to see a toxicity, supporting the fact that C9orf72 pathology relies on all three mechanisms.

We also reported a decrease of synaptic vesicle turnover with decrease frequency and amplitude of mEPCs. This highlighted *C9orf72* LOF as a major contributor in ALS pathogenesis. It unravelled a role for C9orf72 at the synapse as a potential regulator of several components at the synaptic cleft such as SV2 and Rab3a. In addition, our model was also able to recapitulate TDP-43 aggregation indicating a possible regulation between C9orf72 and TDP-43 explaining TDP-43 mislocalization and aggregation.

Lastly, by reporting a downregulation of the calpastatin, we were able to treat and rescue phenotypes in our model using calpain inhibitor, suggesting a potential therapeutic avenue. This pathway being dysregulated in other neurodegenerative diseases, it could lead to useful therapy beyond ALS.

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6 APPENDIX I: LIST OF OTHER PUBLICATIONS

1. Butti Z and Patten SA. RNA dysregulation in amyotrophic lateral sclerosis. Frontiers in genetics. (2019). doi: 10.3389/fgene.2018.00712 (Appendix II).

2. Fortier G, Butti Z and Patten SA. Modelling C9orf72-related amyotrophic lateral sclerosis in zebrafish. Biomedicines. (2020). doi: 10.3390/biomedicines8100440 (Appendix III).

3. Preville M, Lissouba A, Butti Z, Giacomotto J, Parker JA and Patten SA. Modelling SMA in zebrafish: A robust in vivo model for screening candidate therapeutic compounds. (Manuscript in preparation).

7 APPENDIX II: RNA DYSREGULATION IN AMYOTROPHIC LATERAL SCLEROSIS

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7.1 Abstract

Amyotrophic lateral sclerosis (ALS) is the most common adult-onset motor neuron disease and is characterized by the degeneration of upper and lower motor neurons. It has become increasingly clear that RNA dysregulation is a key contributor to ALS pathogenesis. The major ALS genes SOD1, TARDBP, FUS, and C9orf72 are involved in aspects of RNA metabolism processes such as mRNA transcription, alternative splicing, RNA transport, mRNA stabilization, and miRNA biogenesis. In this review, we highlight the current understanding of RNA dysregulation in ALS pathogenesis involving these major ALS genes and discuss the potential of therapeutic strategies targeting disease RNAs for treating ALS.

7.2 Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive and fatal neurodegenerative disorder of motor function. It is characterized by the selective degeneration of the lower and upper motor neurons. Among the symptoms of this disease are progressive muscle weakness and paralysis, swallowing difficulties and breathing impairment due to respiratory muscle weakness that ultimately causes death, usually within 2–5 years following clinical diagnosis (Kiernan *et al.*, 2011). Though most cases of ALS are sporadic, some families (10%) demonstrate a clinically indistinguishable form of ALS with clear Mendelian inheritance and high penetrance (Pasinelli *et al.*, 2006). Treatments to slow the progression of ALS to date remains Riluzole (Bensimon *et al.*, 1994) and Edaravone (Abe *et al.*, 2014) but they are only modestly effective. However, in the past couple years, there has been a real encouragement in witnessing potentially efficacious treatments, such as Masitinib and Pimozide (Patten *et al.*, 2017; Petrov *et al.*, 2017; Trias *et al.*, 2016) claiming to demonstrate clinical benefit. Furthermore, RNA-targeted therapies are currently intensively being evaluated as potential strategies for treating this ALS (Mathis *et al.*, 2018; Schoch *et al.*, 2017). There is indeed hope to have new and potentially more effective treatment options available for ALS in the near future.

Mutations in over more than 20 genes contribute to the aetiology of ALS (Chia *et al.*, 2018) (Table 1). Amongst these genes, the major established causal ALS genes are SOD1 (Cu-Zn superoxide dismutase 1), TARDBP (transactive response DNA Binding protein 43kDa), FUS (fused in sarcoma) and hexanucleotide expansion repeat in Chromosome 9 Open Reading Frame 72 (C9ORF72). These genetic discoveries have led to the development of animal models (Julien *et al.*, 2006; Kabashi *et al.*, 2010; Patten *et al.*, 2014; Picher-Martel *et al.*, 2016) that permitted the identification of key pathobiological insights. Currently, RNA dysregulation appears to be a major

contributor to ALS pathogenesis. Indeed, TDP-43 and FUS are deeply involved in RNA processing such as transcription, alternative splicing and microRNA (miRNA) biogenesis (Buratti *et al.*, 2004; Polymenidou *et al.*, 2012). Mutations in C9ORF72, lead to a toxic mRNA gain of function through RNA foci formation, and the subsequent sequestration in stress granules and altered activity of RNA-binding proteins (Barker *et al.*, 2017). In addition to the major ALS genes, other ALS genes including ataxin-2 (ATXN2) (Ostrowski *et al.*, 2017), TATA-box binding protein associated factor 15 (TAF15) (Ibrahim *et al.*, 2013), heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1) (Dreyfuss *et al.*, 1993), heterogeneous nuclear ribonucleoprotein A2 B1 (hnRNPA2 B1) (Alarcon *et al.*, 2015), matrin 3 (MATR3) (Coelho *et al.*, 2015), Ewing's sarcoma breakpoint region 1 (EWSR1) (Duggimpudi *et al.*, 2015), T-cell-restricted intracellular antigen-1 (TIA1) (Forch *et al.*, 2000), senataxin (SETX) and angiogenin (ANG) (Yamasaki *et al.*, 2009), play critical role in RNA processing (Table 1).

In this review, we focus on the four major ALS-associated genes (SOD1, TARDBP, FUS, and C9orf72) and present how they play critical roles in various RNA pathways. We particularly highlight recent developments on the dysregulation of RNA pathways (Figure 1) as a major contributor to ALS pathogenesis and discuss the potential of RNA-targeted therapies for ALS.

7.3 TAR DNA binding protein (TDP-43)

A major advance in our understanding of cellular mechanisms in ALS came from the identification of causative mutations in the TARDBP gene (Kabashi *et al.*, 2008; Sreedharan *et al.*, 2008). This gene encodes for the evolutionarily conserved RNA/DNA binding protein, TDP-43. It is a protein that is normally nuclear, however, in cases of TARDBP mutations, it is mislocalized to the cytoplasm and forms aggregates (Van Deerlin *et al.*, 2008; Winton *et al.*, 2008b). It is found in the pathological aggregates in motor neurons in the majority of cases of ALS (Neumann *et al.*, 2006). It is believed that TDP-43 aggregation leads to a gain of toxicity and its nuclear depletion results to a loss of function of TDP-43. Indeed, several studies have demonstrated that either overexpression or knockdown of TDP-43 causes neurodegeneration and ALS phenotypes (Iguchi *et al.*, 2013; Kabashi *et al.*, 2010; Stallings *et al.*, 2010; Yang *et al.*, 2014). For instance, the expression of the mutant TDP-43 A315T in the C. elegans' GABAergic motor neurons results in age-dependent motility defects and neurodegeneration (Vaccaro *et al.*, 2012). In drosophila, overexpression of TDP-43 in motor neurons was found to cause cytoplasmic accumulation of TDP-43 aggregates, neuromuscular junction (NMJ) morphological defects and cell death (Li *et al.*, 2010). Similarly, the loss of TDP-43 reduced locomotion and lifespan (Diaper *et al.*, 2013;

Feiguin *et al.*, 2009). Implications of TDP-43 loss and toxic gain-of-function in impaired motility, neurodegeneration and survival were further confirmed in higher model systems such as the zebrafish (Kabashi *et al.*, 2010) and mice (Iguchi *et al.*, 2013; Wegorzewska *et al.*, 2009). Altogether, these reports strongly suggest that alterations in the level of TDP-43 are detrimental to neuronal function and survival.

TDP-43 contains two RNA recognition motifs (RRM1-2), a glycine rich domain in the C-terminus and nuclear localization and export signals (NLS and NES) (Buratti *et al.*, 2001; Winton *et al.*, 2008a). TDP-43 plays a major role in multiple steps of RNA processing such as splicing, RNA stability and mRNA transport (Buratti *et al.*, 2008). For instance, TDP43 has been shown to bind to mRNA and regulate the expression of other proteins implicated in ALS and other neurodegenerative diseases such as FUS, Tau, ATXN 2 and progranulin (Polymenidou *et al.*, 2011; Sephton *et al.*, 2011; Tollervey *et al.*, 2011). This suggests that TDP-43 may be a central component in the pathogenesis of several neurodegenerative conditions (Polymenidou *et al.*, 2011). By RNA-seq analysis, Polymenidou et al. (2011) reported that TDP-43 is required for regulating the expression of 239 mRNAs, many of those encoding synaptic proteins. Several independent studies have corroborated that TDP-43 plays an important role in regulating genes involved in synaptic formation and function and in the regulation of neurotransmitter processes (Chang *et al.*, 2014; Colombrita *et al.*, 2012; Godena *et al.*, 2011; Narayanan *et al.*, 2013; Sephton *et al.*, 2011). Examples of such genes are neurexin (NRXN1-3) (Polymenidou *et al.*, 2011), neuroligin (NLGN1-2) (Polymenidou *et al.*, 2011), scaffolding protein Homer2 (Sephton *et al.*, 2011), microtubule-associated protein 1B (MAP1B) (Coyne *et al.*, 2014), GABA receptors subunits (GABRA2, GABRA3) (Narayanan *et al.*, 2013), AMPA receptor subunits (GRIA3, GRIA4) (Narayanan *et al.*, 2013; Sephton *et al.*, 2011), syntaxin 1B (Narayanan *et al.*, 2013), and calcium channel cacophony (Chang *et al.*, 2014). The development of TDP-43 animal models has offered the opportunity to explore synaptic alterations in ALS (Armstrong *et al.*, 2013a; Feiguin *et al.*, 2009; Handley *et al.*, 2017) and continuous efforts are being made to identify compounds that can facilitate synaptic transmission in ALS (Patten *et al.*, 2017). Armstrong and Drapeau (2013) reported that expression of mutant TARDPG348C mRNA in zebrafish resulted in impaired synaptic transmission, reduced frequency of miniature endplate currents (mEPCs) and reduced quantal transmission. Remarkably, they also demonstrated that all these synaptic dysfunction features in their zebrafish TARDBP mutant were stabilized by chronic treatment the L-type calcium channel agonists (Armstrong *et al.*, 2013a). In drosophila neurons, TDP-43 depletion was shown to reduce dendritic branching as well as synaptic formation (Feiguin *et al.*, 2009; Lu *et al.*, 2009b). Overexpression or knocking down TDP-43 in cultured mammalian neurons also led to

reduced dendritic branching (Herzog *et al.*, 2017). In TDP-43A315T mice, Handley et al. (2017) showed that expression of mutant TDP-43 alters dendritic spine development, spine morphology and neuronal synaptic transmission. Collectively, these independent studies on several model systems, suggest that TDP-43 may play an important role in neuronal morphology, synaptic transmission and neuronal plasticity likely via regulation of RNA processing of various synaptic genes (Chang *et al.*, 2014; Colombrita *et al.*, 2012; Godena *et al.*, 2011; Narayanan *et al.*, 2013; Sephton *et al.*, 2011).

TDP-43 is also known to act as a splicing regulator to reduce its own expression level by binding to the 3′ UTR of its own pre-mRNA (Ayala *et al.*, 2011). Additionally, it functions as a splicing factor whose depletion or overexpression can affect the alternative splicing of specific targets (Polymenidou *et al.*, 2011; Tollervey *et al.*, 2011). Indeed, the alternative splicing of several genes were reported to be altered in human CNS tissues from TDP-43 ALS cases (Shiga *et al.*, 2012; Yang *et al.*, 2014). For instance, the level of the polymerase delta interacting protein 3 (POLDIP3) variant-2 mRNA (lacking exon 3) was significantly increased in the CNS of ALS patients with ALS, while that of variant-1 mRNA remained unchanged (Shiga *et al.*, 2012). This was consistent with findings that TDP-43 directly regulates the inclusion of exon 3 of POLDIP3 and that depletion of TDP-43 in cell culture models increased variant-2 mRNA (Shiga *et al.*, 2012). TDP-43 has also been shown to regulate splicing of the cystic fibrosis transmembrane regulator (CFTR) gene and controls exon skipping by within the pre-mRNA (Buratti *et al.*, 2004). Importantly, it controls the alternative splicing of apolipoprotein AII (APOAII) (Mercado *et al.*, 2005) and survival of motor neuron (SMN) transcripts (Bose *et al.*, 2008). Specifically, TDP-43 was shown to enhance the inclusion of exon 7 during the maturation of human SMN2 pre-mRNA, which results to an increase in full-length SMN2 mRNA level in neurons (Bose *et al.*, 2008). Furthermore, recently TDP-43 was shown to bind to HNRNPA1 pre-mRNA to modulate its alternative splicing (Deshaies *et al.*, 2018). TDP-43 depletion resulted in exon7B inclusion, culminating in a longer hnRNAP A1B isoform that is aggregation-prone and cytotoxic (Deshaies *et al.*, 2018). Collectively, these studies demonstrated that loss of TDP-43 results to alterations in alternative splicing of many genes and some of which, for example HNRNPA1, can contribute to cellular vulnerability. It would be interesting further to investigate the contribution of the alteration of splicing of these genes (POLDIP3, CFTR, APOAII, SMN2, HNRNPA1) to the pathogenesis of ALS.

TDP-43 is actively transported along axons and co-localizes with other well-known transport RNA binding proteins close to synaptic terminals (Narayanan *et al.*, 2013; Wang *et al.*, 2008b). It was reported that TDP-43 mutations impair mRNA transport function in vivo and in vitro (Alami *et al.*,

2014). In addition to a role in mRNA transport, TDP-43 also acts as a regulator of mRNA stability (Fiesel *et al.*, 2011; Strong *et al.*, 2007). It was shown to directly interacts with the 3′ UTR of neurofilament light chain (NFL) mRNA to stabilize it (Strong *et al.*, 2007) and associates with futsch/MAP1B mRNA in Drosophila to regulates its localization and translation (Coyne *et al.*, 2014). Particularly, TDP-43 was found to interact with 14-3-3 protein subunits to modulate the stability of the NFL mRNA (Volkening *et al.*, 2009). Abnormal regulation of NFL mRNA has been observed in ALS patients (Wong *et al.*, 2000) and disruption of NFL mRNA stoichiometry leads to motor neuron death and symptoms of ALS in animal models (Julien *et al.*, 1995; Xu *et al.*, 1993). It is, thus, very likely that TDP-43 mutations may cause motor neuron degeneration by interfering with RNA processing of NFL mRNA.

Other important identified targets regulated by TDP-43 at mRNA level that may play a role in disease are G3BP (McDonald *et al.*, 2011) and TBC1D1 (Stallings *et al.*, 2013). G3BP is an essential component of stress granules, which are cytoplasmic non-membrane organelles that store translationally arrested mRNAs that accumulate during cellular stress (Kedersha *et al.*, 2007). Stress granules consists of polyadenylated mRNAs, translation initiation factors (e.g., eIF3, eIF4E, and eIF4G), small ribosomal subunits and a numerous RNA-binding proteins (Protter *et al.*, 2016). TDP-43 is recruited to stress granules in cellular models upon exposure to different stressors (Bentmann *et al.*, 2012; Colombrita *et al.*, 2009; Liu-Yesucevitz *et al.*, 2010). Importantly, cytosolic TDP-43 mutants are more efficiently recruited to stress granules upon cellular stress compared to nuclear wild-type TDP-43 (Liu-Yesucevitz *et al.*, 2010). Prolonged stress is thought to promote sequestration of TDP-43 and their mRNA targets in stress granules; thereby inhibiting translation and potentially contributing to ALS progression (Ramaswami *et al.*, 2013).

7.4 Fused in sarcoma (FUS)

Mutations in FUS are detected in 4–5% of familial ALS patients as well as in sporadic ALS (Corrado *et al.*, 2010; DeJesus-Hernandez *et al.*, 2010; Kwiatkowski *et al.*, 2009; Vance *et al.*, 2009). FUS is an RNA/DNA-binding protein of 526 amino acids, consisting of an RNA-recognition motif, a SYGQ (serine, tyrosine, glycine and glutamine)-rich region, several RGG (arginine, glycine and glycine)-repeat regions, a C2C2 zinc finger motif and a nuclear localization signal (NLS) (Iko *et al.*, 2004). C-terminal ALS FUS mutations disrupt the NLS region and the nuclear import of FUS; resulting in cytoplasmic accumulation (Kwiatkowski *et al.*, 2009; Vance *et al.*, 2009).

Similarly to TDP-43, FUS plays multiple roles in RNA processing by directly binding to RNA. Using CLIP-based methods, several groups have identified thousands of RNA targets bound by FUS in various cell lines (Colombrita *et al.*, 2012; Hoell *et al.*, 2011; Ishigaki *et al.*, 2012), and brain tissues (Lagier-Tourenne *et al.*, 2012; Rogelj *et al.*, 2012). Interestingly, FUS was identified in spliceosomal complexes (Rappsilber *et al.*, 2002; Zhou *et al.*, 2002) and interacting with several key splicing factors (such as hnRNP A1, YB-1) (Kamelgarn *et al.*, 2016; Meissner *et al.*, 2003; Rapp *et al.*, 2002) as well as with the U1 snRNP (Yamazaki *et al.*, 2012; Yu *et al.*, 2015). FUS regulates splicing events for neuronal maintenance and survival (Lagier-Tourenne *et al.*, 2012). Given that FUS plays an essential role in splicing regulation, the consequence of its loss of function in ALS on RNA splicing has been immensely investigated (Lagier-Tourenne *et al.*, 2012; Reber *et al.*, 2016; Zhou *et al.*, 2013b). For instance, Reber et al. (2016) showed by mass spectrometric analysis that minor spliceosome components are highly enriched among the FUS-interacting proteins. They further reported that FUS interacts with the minor spliceosome and directly regulates the removal of minor introns (Reber *et al.*, 2016). Moreover, the FUSP525L ALS mutation, which destroys the NLS and results in cytoplasmic retention of FUS (Dormann *et al.*, 2010), inhibits splicing of minor introns and causes mislocalization of the minor spliceosome components U11 and U12 snRNA to the cytoplasm and inhibits splicing of minor introns (Reber *et al.*, 2016). Loss of function of FUS led to splicing changes in more than 300 genes mice brains (Lagier-Tourenne *et al.*, 2012) and importantly a vast majority minor intron containing mRNAs was altered (Reber *et al.*, 2016). Corroborating the results with mouse brain, many minor intron-containing genes were found to be downregulated in FUS-depleted SH-SY5Y cells (Reber *et al.*, 2016). FUS depletion has been shown to affect minor intron containing genes that are important for neurogenesis (PPP2R2C), dendritic development (ACTL6B) and action potential transmission in skeletal muscles (SCN8A and SCN4A) (Reber *et al.*, 2016) and may contribute to ALS pathogenesis. FUS has also been shown to regulate alternative splicing of genes related to cytoskeletal organization, axonal growth and guidance such as the microtubule-associated protein tau (MAPT) (Ishigaki *et al.*, 2012; Orozco *et al.*, 2012; Rogelj *et al.*, 2012), Netrin G1 (NTNG1) (Rogelj *et al.*, 2012), neuronal cell adhesion molecule (NRCAM) (Nakaya *et al.*, 2013; Rogelj *et al.*, 2012) and the actin-binding LIM (ABLIM1) (Nakaya *et al.*, 2013). For example, FUS knockdown has been shown to promote inclusion of exon 10 in the MAPT/tau protein and to significantly cause shortened axon length and growth cone enlargement (Orozco *et al.*, 2012). Loss of function of FUS altered MAPT/tau isoform expression and likely disturbed cytoskeletal function impairing axonal growth and maintenance. Interestingly, axon retraction and denervation

are early events in ALS (Boillee *et al.*, 2006; Nijssen *et al.*, 2017). Disruption of cytoskeleton function may thus play an important role in neurodegeneration in ALS.

Besides its functions in splicing, FUS has been proposed to regulate transcription by RNA polymerase II (RNAP2), RNA polymerase III (RNAP3) or cyclin D1 (Brooke *et al.*, 2011; Schwartz *et al.*, 2012; Tan *et al.*, 2010; Tan *et al.*, 2012; Wang *et al.*, 2008c). For instance, transcriptomic analyses showed that knockdown of FUS results in differential expression several genes (Lagier-Tourenne *et al.*, 2012; Nakaya *et al.*, 2013) including many mRNAs encoding proteins important for neuronal function. Transcriptome changes have also been observed in human motoneurons obtained from FUS mutant induced pluripotent stem cells (IPSCs) (De Santis *et al.*, 2017) and transgenic FUS knockin mice (Scekic-Zahirovic *et al.*, 2016). Alterations in the expression of several genes involved in pathways related to cell adhesion, apoptosis, synaptogenesis and other neurodegenerative diseases were reported in these FUS models (De Santis *et al.*, 2017; Fujioka *et al.*, 2013; Scekic-Zahirovic *et al.*, 2016). Among these genes TAF15, which is mutated in some case of ALS (Couthouis *et al.*, 2011), has been found to be upregulated in several ALS FUS models including human mutant IPSC derived motoneurons (De Santis *et al.*, 2017), FUS knockout and knockin mouse (Kino *et al.*, 2015; Scekic-Zahirovic *et al.*, 2016). However, it remains to be determined whether TAF15 upregulation upon FUS loss- or toxic gain- of function contributes to ALS pathogenesis.

FUS is also incorporated into stress granules under cellular stress conditions (Sama *et al.*, 2013). Sequestration of FUS and its protein partners into these cytoplasmic organelles appears to contribute to ALS pathogenesis (Yasuda *et al.*, 2013). An example of such a protein partner is Pur-alpha, which co-localizes with mutant FUS and becomes trapped in stress granules in stress conditions, as reported in ALS patient cells carrying FUS mutations (Daigle *et al.*, 2016; Di Salvio *et al.*, 2015). It has been shown that FUS physically interacts with Pur-alpha. In vivo expression of Pur-alpha in Drosophila significantly exacerbates the neurodegeneration caused by mutated FUS. Conversely, Di Salvio et al. (2015) showed that the downregulation of Pur-alpha in neurons expressing mutated FUS significantly improves fly climbing activity. It was subsequently demonstrated that overexpression Pur-alpha inhibits cytoplasmic mislocalization of mutant FUS and promotes neuroprotection (Daigle *et al.*, 2016). However, the function of Pur-alpha in regulating ALS pathogenesis remains elusive.

7.5 Superoxide dismutase 1 (SOD1)

Unlike TDP43 and FUS, SOD1 does not contain RNA-binding motifs, however, several reports have demonstrated a potential role of mutant SOD1 in regulating RNA metabolism (Chen *et al.*, 2014; Lu *et al.*, 2009a; Lu *et al.*, 2007; Menzies *et al.*, 2002). Particularly, mutant SOD1 can bind mRNA species such as vascular endothelial growth factor (VEGF) and NFL and negatively affects their expression, stabilization and function (Chen *et al.*, 2014; Lu *et al.*, 2009a; Lu *et al.*, 2007; Menzies *et al.*, 2002). More precisely, mutant SOD1 can directly bind to specific adenylate- and uridylate-rich stability elements (AREs) located in the 3′ UTR of transcripts of VEGF (Lu *et al.*, 2007) and NFL (Chen *et al.*, 2014). It is believed that such a gain of abnormal protein–RNA interactions can be caused by SOD1 misfolding that results in the exposure of polypeptide portions with the ability to bind nucleic acids (Kenan *et al.*, 1991; Tiwari *et al.*, 2005).

Binding of mutant SOD1 to the 3′ UTR of the VEGF mRNA results in the sequestration of other ribonucleoproteins such as TIAR and HuR into insoluble aggregates. These interactions, which are specific to mutant SOD1, result in decline levels of VEGF mRNA, impairment of HuR function and ultimately hampering their neuroprotective actions during stress responses (Lu *et al.*, 2009a; Lu *et al.*, 2007).

In motor neuron-like NSC34 cell lines expressing mutant SOD1 (G37R or G93A), the level of NFL mRNA is significantly reduced (Menzies *et al.*, 2002). Reduction in NFL mRNA levels has also been reported in G93A transgenic mice and human spinal motor neurons from SOD1-ALS cases (Menzies *et al.*, 2002). It is proposed that destabilization NFL mRNA by mutant SOD1, result to altered stoichiometry of neurofilament (NF) subunits and subsequent NF aggregation in motor neurons (Chen *et al.*, 2014). NF inclusion in the soma and proximal axons of spinal motor neurons is a hallmark of ALS pathology (Hirano *et al.*, 1984). In IPSC-derived model of ALS, a reduction of NFL mRNA level has been reported to result in NF aggregation and neurite degeneration (Chen *et al.*, 2014). Altogether, these studies support a pathogenic role for dysregulation of RNA processing in SOD1-related ALS.

Interestingly, SOD1 has been shown to interact with TDP-43 to modulate NFL mRNA stability (Volkening *et al.*, 2009). As mentioned above, TDP-43 was found to directly interact with the 3′ UTR of NFL mRNA to stabilize it (Strong *et al.*, 2007). Altogether, these studies suggest that SOD1 and TDP-43 may act in a possible common action in regulating specific RNA stability. In the case of NFL mRNA, it would be interesting to investigate whether mutant SOD1 dislodges TDP-43 from the NFL mRNA in a manner that would affect its mRNA metabolism and potentially making NF prone to form aggregates.

Furthermore, there have been several transcriptome investigations in SOD1 human samples (D'Erchia *et al.*, 2017), motor neuron-like NSC34 cell culture model (Kirby *et al.*, 2005) and transgenic animals including mice (Bandyopadhyay *et al.*, 2013; Lincecum *et al.*, 2010; Sun *et al.*, 2015), rat (Hedlund *et al.*, 2010) and drosophila (Kumimoto *et al.*, 2013). These studies have reported dysregulation of genes involved in pathways related to the neuroinflammatory and immune response, oxidative stress, mitochondria, lipid metabolism, synapse and neurodevelopment (Bandyopadhyay *et al.*, 2013; D'Erchia *et al.*, 2017; Hedlund *et al.*, 2010; Kumimoto *et al.*, 2013; Lincecum *et al.*, 2010; Sun *et al.*, 2015). However, in these studies it is not clear whether SOD1 directly or indirectly impact the regulation of the differentially expressed genes. In a recent elegant study, Rotem et al. (2017), compared transcriptome changes in SOD1 and TDP-43 models. They found that most genes that were altered in the SOD1G93A model were not dysregulated in the TDP-43A315T model, and vice versa (Rotem *et al.*, 2017). There were, however, a few genes whose expressions were altered in both ALS models (Rotem *et al.*, 2017). These findings are consistent with the ALS pathology, which is distinguishable between the ALS-related SOD1 phenotype and the TDP-43 phenotype. Although different cellular pathways are likely activated by SOD1 versus TDP-43, it is very plausible that they ultimately convergence onto common targets to result in similar motor neuron toxicity and ALS phenotype.

7.6 C9orf72 intronic expansion

In 2011, a large GGGGCC hexanucleotide repeat expansion in the first intron or promoter region of the C9orf72 gene has been discovered as a new cause of ALS (DeJesus-Hernandez *et al.*, 2011; Renton *et al.*, 2011). C9orf72 repeat expansion mutations account for about 50% of familial ALS and 5–10% of sporadic ALS (Majounie *et al.*, 2012). It remains a topic of debate whether the repeat expansion in C9orf72 causes neurodegeneration primarily through a toxic gain of function, loss of function, or both. The C9orf72 repeat expansion is transcribed in both the sense and antisense directions and leads to accumulations of repeat-containing RNA foci in patient tissues (Gendron *et al.*, 2013). The formation of RNA foci facilitates the recruitment of RNA-binding proteins, causes their mislocalization and interferes with their normal functions (Donnelly *et al.*, 2013; Gitler *et al.*, 2016; Lee *et al.*, 2013; Simon-Sanchez *et al.*, 2012). Indeed, RNA foci may bind RNA binding protei; ns and alter RNA metabolism (Donnelly *et al.*, 2013; Lee *et al.*, 2013; Mori *et al.*, 2013b)For example, Mori et al. (2013a) and Hutvagner et al. (2001) showed that RNA foci can sequester hnRNP-A3 and repress its RNA processing function (Hutvagner *et al.*, 2002; Mori *et al.*, 2013b). Aborted transcripts containing the repeat can also disrupt nucleolar function (Haeusler *et al.*, 2014). Importantly, these foci can sequester nuclear proteins such as TDP-43

and FUS, impacting expression of their RNA targets and culminating in a range of RNA misprocessing events. Other RNA binding proteins binding to RNA foci include hnRNP A1, hnRNP-H, ADARB2, Pur-α, ASF/SF2, ALYREF and nucleolin (Cooper-Knock *et al.*, 2014; Donnelly *et al.*, 2013; Haeusler *et al.*, 2014; Lee *et al.*, 2013; Sareen *et al.*, 2013; Xu *et al.*, 2013). Antisense oligonucleotides (ASOs) targeting the C9orf72 repeat expansion suppress RNA foci formation, attenuate sequestration of specific RNA-binding proteins and reverse gene expression alterations in C9orf72 ALS motor neurons derived from IPSCs (Donnelly *et al.*, 2013; Lagier-Tourenne *et al.*, 2013).

Additionally, simple dipeptide repeats (poly-GA, poly-GP, poly-GR, poly-PA, and poly-PR) can be generated by repeat-associated non-ATG-dependent (RAN) translation of both the sense and antisense strands that have a variety of toxic effects (Ash *et al.*, 2013; Mori *et al.*, 2013c). Poly-PR and poly-GR can alter the splicing patterns of specific RNAs. For example, poly-PR has been shown to cause exon-skipping in RAN and PTX3 RNA (Kwon *et al.*, 2014). Dipeptides repeat proteins have also been found to be toxic by creating aggregates sequestrating cytoplasmic proteins (Freibaum *et al.*, 2017). Poly-GR dipeptide co-localizes with several ribosomal subunits and with a transcription factor eIF3η (Zhang *et al.*, 2018d). This suggests a ribosomal dysfunction, which implies a defect in RNA translation. In line with these findings, a recent report demonstrated that poly-PR co-localizes with the nucleolar protein, nucleophosmin, and reduces the expression of several ribosomal RNA (Suzuki *et al.*, 2018). Suzuki et al. (2018) further showed that the reduction in the expression of ribosomal RNA results in neuronal cell death and this could be rescued by overexpression of an accelerator of ribosome biogenesis, Myc (Suzuki *et al.*, 2018). RNA sequencing reveals that more than 6,000 genes are up or down regulated in mice that express the dipeptide construct in the brain (Zhang *et al.*, 2018d). Other findings show that poly-PR dipeptide binds nuclear pores channels blocking the import and export of molecules. The dipeptide actually binds the nucleoporin proteins Nup54 and Nup98 that rim the central channel of the pore (Shi *et al.*, 2017). The accumulation of poly-PR dipeptide at the nuclear pore was found to correlate with defect in nuclear transport of RNA and protein, which is consistent with previous findings (Freibaum *et al.*, 2015; Zhang *et al.*, 2015).

The last proposed mechanism involved in ALS pathogenesis is a haploinsufficiency due to the expansion of repetition leading to a decreased transcription of the gene and consequently to a decrease of its translation (Ciura *et al.*, 2013). Studies have demonstrated that C9orf72 expansion repeat can interfere with transcription or splicing of C9orf72 transcripts (Haeusler *et al.*, 2014; Highley *et al.*, 2014; Mori *et al.*, 2013c). It has also been proposed that the C9orf72 expansion

repeat could disrupt the C9orf72 promoter activity thereby reducing its expression (Gijselinck *et al.*, 2016). Several studies have demonstrated alterations in the C9orf72 ALS transcriptome (Donnelly *et al.*, 2013; Prudencio *et al.*, 2015; Selvaraj *et al.*, 2018). Interestingly, a recent article reported an increased expression of the calcium-permeable GluA1 AMPA receptor subunit in motoneurons derived from IPSC of patients with C9orf72 mutations (Selvaraj *et al.*, 2018). This alteration in AMPA receptor composition led to an enhanced motoneuron vulnerability to AMPA-induced excitotoxicity (Selvaraj *et al.*, 2018). It remains to be determined whether the increased expression of GluA1 AMPA subunit is related to reduced levels of C9orf72, RNA foci and/or dipeptide repeats.

C9orf72 has also been showed to be involved in the generation of stress granules (Maharjan *et al.*, 2017) and sequestering other RNA binding proteins that are involved in nucleo-cytoplasmic transport (Zhang *et al.*, 2018b; Zhang *et al.*, 2015). It has been found that stress granules observed in C9orf72 mutants co-localizes with Ran GAP (Zhang *et al.*, 2018b; Zhang *et al.*, 2015); which is known to activate Ran GTPase. This GTPase in involved in nucleo-cytoplasmic transport. It has also been published that expressing Ran GAP rescues the age-related motor defects in flies expressing the GGGGCC repeats (Zhang *et al.*, 2018a). Very recently, it has also been reported that one of the dipeptide generated by the expansion has a role in formation of these stress granules (Zhang *et al.*, 2018d). Moreover, importins and exportins are sequestered in stress granules; which also implies that protein transport in altered (Zhang *et al.*, 2018b).

These toxic gain- or loss-of function mechanisms are thought to be all involved in synergy in ALS pathogenesis and it can be summed up that that altered RNA processing plays a key role in C9orf72-mediated toxicity through two ways. The first is altered processing of the expanded C9orf72 transcript itself, in terms of altered transcription, splicing defects, nuclear aggregation and non-conventional translation (Barker *et al.*, 2017). The second involves downstream and indirect changes in RNA processing of other transcripts. A thorough understanding of RNA metabolism dysregulation could definitely bring a major enlightenment on how C9orf72 mutation leads to ALS and provide insights on therapeutic targets.

7.7 Dysregulation of microRNA (miRNA) in ALS

Multiple mechanisms control the proper levels of RNA and subsequent protein expression; among these are microRNAs (miRNAs) (Catalanotto *et al.*, 2016). They are endogenous small non-coding RNAs (approximately 22 nucleotides in length) that are initially transcribed by the RNA polymerase II as primary miRNA (pri-miRNAs) transcripts. These pri-miRNAs are processed into

precursor miRNAs (pre-miRNAs) by the nuclear ribonuclease III (RNase III), DROSHA, and the double-stranded RNA-binding protein, DGCR8, which anchors DROSHA to the pri-miRNA transcript (Denli *et al.*, 2004; Lee *et al.*, 2003). Pre-miRNA is then exported into the cytoplasm by exportin-5 (Yi *et al.*, 2003), where it is processed into a mature miRNA by the DICER enzyme (Hutvagner *et al.*, 2002; Ketting *et al.*, 2001). The mature miRNA is then incorporated with a ribonucleoprotein (RNP) complex with argonaute (AGO) proteins to form the RNA-induced silencing complex (RISC) (Hammond *et al.*, 2001; Kawamata *et al.*, 2010; Schwarz *et al.*, 2003), which mediates inhibition of translation and/or mRNA degradation of targeted transcripts that are complementary to the miRNA (Hutvagner *et al.*, 2002; Yekta *et al.*, 2004). The recognition of mRNAs by miRNAs occurs through base-pairing interactions within the 3′-untranslated region (UTR) of the targeted mRNAs. Besides their well-known gene silencing functions, miRNAs can also induce up-regulation of their targets (Lin *et al.*, 2011a; Truesdell *et al.*, 2012; Vasudevan, 2012; Vasudevan *et al.*, 2007).

MiRNAs play important roles in several biological processes such as cell proliferation (Chen et al., 2006), cell differentiation (Naguibneva *et al.*, 2006), apoptosis (Matsushima *et al.*, 2011), and patterning of the nervous system (Johnston *et al.*, 2003). Interestingly, several miRNAs have been particularly shown to be essential for motor neuron development and survival (see review, (Haramati *et al.*, 2010). For example, in developing chick, it was demonstrated that the activation of the miRNA miR9 is necessary to suppress the expression of the transcription factor onecut1, which in turn helps to drive differentiation of neural progenitor cells into spinal motor neurons (Luxenhofer *et al.*, 2014). It is believed that several miRNAs work in concert to establish motor neuron identity. Indeed, in addition to miR9, other miRNAs such as miR-128 (Thiebes *et al.*, 2015), miR-196 (Asli *et al.*, 2010), miR-375 (Bhinge *et al.*, 2016) have been shown to play a role in motor neuron differentiation and localization. Loss of DICER function within progenitor cells results in aberrant motor neuron development while its loss in motor neuron leads to progressive motor neuron degeneration (Chen *et al.*, 2012; Haramati *et al.*, 2010). Furthermore, miRNAs are important players for NMJ function, synaptic plasticity and for maintaining cytoskeletal integrity (see review, (Hawley *et al.*, 2017).

The ALS genes, TDP-43 and FUS, were identified in a protein complex with RNAse III DORSHA and shown to play a role in miRNA biogenesis (Da Cruz *et al.*, 2011; Freibaum *et al.*, 2010). TDP-43, in particular was shown to associate with proteins involved in the cytoplasmic cleavage of pre-miRNA mediated by the DICER enzyme (Freibaum *et al.*, 2010). It is thus to no surprise that dysregulation of miRNAs has been observed in ALS (Dini Modigliani *et al.*, 2014; Eitan *et al.*,

2016; Li *et al.*, 2013; Zhang *et al.*, 2013). Indeed, mutations in TARDBP result in differential expression of miRNAs – miR-9, miR-132, miR-143, and miR-558 (Kawahara *et al.*, 2012; Zhang *et al.*, 2013). Interestingly, the expression of several of these miRNAs (miR-9, miR-132, miR-143) and including others (such as miR-125, miR-192) are altered upon FUS depletion (Morlando *et al.*, 2012). MiR-9 expression is also found to be upregulation in mutant SOD1 mice (Zhou *et al.*, 2013a). These dysregulated miRNAs are essential for motor neuron development and maintenance (Luxenhofer *et al.*, 2014; Otaegi *et al.*, 2011), axonal growth (Dajas-Bailador *et al.*, 2012; Kawahara *et al.*, 2012) and synaptic transmission (Edbauer *et al.*, 2010; Sun *et al.*, 2012). Thus, these miRNA alterations likely contribute to the pathological phenotype observed in ALS.

Additionally, depletion of TDP-43 in cell culture systems has also been shown to change the total miRNA expression profile (Buratti *et al.*, 2010). A similar observation was recently observed in motoneurons progenitors derived from human ALS IPSCs (Rizzuti *et al.*, 2018). Particularly, it was reported that 15 miRNAs were dysregulated including disease-relevant miR-34a and miR504, which are known to be, implicated synaptic vesicle regulation and cell survival (Rizzuti *et al.*, 2018). Additionally, another important miRNA, namely microRNA-1825, was found to be downregulated in CNS of both sporadic and familial ALS patients (Helferich *et al.*, 2018). Interestingly, reduced levels of microRNA-1825 was demonstrated to cause a translational upregulation of tubulin-folding cofactor b (TBCB) which consequently to depolymerization and degradation of tubulin alpha-4A (TUBA4A), which is encoded by a known ALS gene (Helferich *et al.*, 2018).

In several repeats diseases such as myotonic dystrophy, fragile X tremor and ataxia syndrome, toxic RNA from expansion repeats cause widespread RNA splicing abnormalities, degeneration of affected tissues (Miller *et al.*, 2000) and alter miRNA processing (Sellier *et al.*, 2013). Since its discovery, C9orf72 GGGGCC expansion repeat was also questioned as a disruptor of miRNA processing. Recently, the DROSHA protein was found to be mislocalized in dipeptide repeat protein-aggregates in frontal cortex and cerebellum C9orf72 ALS/FTLD patients (Porta *et al.*, 2015). An involvement of the miRNA pathway in motor neuron impairment in ALS is evident and further investigations on miRNAs dysregulation in ALS pathogenesis could eventually lead to the identification of therapeutic targets.

7.8 RNA targeted therapeutics for ALS

Our understanding of RNA biology has expanded tremendously over the past decades, resulting in new approaches to engage RNA as a therapeutic target. More precisely, RNA-targeted

therapeutics have been developed to mediate the reduction or expression of a given target RNA by employing mechanisms such as RNA cleaving, modulation of RNA splicing, inhibition of mRNA translation into protein, inhibition of miRNA binding sites, increasing translation by targeting upstream open reading frames and disruption of RNA structures regulating RNA stability (Fellmann *et al.*, 2014; Havens *et al.*, 2016; Liang *et al.*, 2016; Robertson *et al.*, 2010; Vickers *et al.*, 2014). Therapeutics that directly target RNAs are promising for a broad spectrum of disorders, including the neurodegenerative diseases (Scoles *et al.*, 2018)and are currently under evaluation as potential strategies for treating ALS. The RNA therapeutics approaches include RNA interference (RNAi) and ASOs (Figure 2), both bind to their target nucleic acid via Watson-Crick base pairing and cause degradation of or inactivate the targeted mRNA (Burnett *et al.*, 2012). Recently, application of innovative drug discovery approaches has showed that targeting RNA with bioactive small molecules is achievable (Bernat *et al.*, 2015; Disney, 2013). A few researchers including us are currently exploiting such a new type of RNA-targeted therapeutics to search for RNA-targeted small molecules as C9orf72 ALS therapeutics.

7.8.1 RNA interference

RNAi is an endogenous cellular mechanism to regulate mRNA. It operates sequence specifically and post-transcriptionally via the RISC (Carthew *et al.*, 2009). Methods of mediating the RNAi effects are via small interfering RNA (siRNA), short hairpin RNA (shRNA), and artificial miRNA) (Chakraborty *et al.*, 2017; Fire *et al.*, 1998; Moore *et al.*, 2010). These approaches can help to reduce the expression of mutant (toxic) gene and can provide significant therapeutic benefit in treating ALS and other neurodegenerative disease implicating aberrant accumulation of misfolded proteins.

The challenge of using siRNA for treating ALS is that it has to be designed to have the specificity and ability to reduce the aberrant mutant protein while leaving wild-type protein intact. Attempts were made to design siRNA, which could recognize just a single nucleotide alternation to selectively suppress mutant SOD1 (particularly G93A) expression leaving wild-type SOD1 intact (Wang *et al.*, 2008a; Yokota *et al.*, 2004). The design of siRNA G93A.1 and G93A.2 by Yokota et al. (2004) were found to successfully suppress the expression of approximately 90% of mutant SOD1 G93A. Importantly, both siRNA had virtually little or no effect on wild-type SOD1 expression (Yokota *et al.*, 2004). To achieve long-term expression of siRNA in cells, the use of viral delivery system has proved powerful to provide a continuous delivery and expression of shRNA in sufficient quantities (Bowers *et al.*, 2011). Indeed, diverse viral vectors have been studied such

as adeno-associated virus (AAV), lentivirus (LV), and rabies-glycoprotein-pseudotyped lentivirus (RGP-LV) (Raoul *et al.*, 2005; Wu *et al.*, 2009). Recombinant AAVs are currently the choice of RNAi treatment vehicle for neurological diseases because they are non-pathogenic and safe (Maguire *et al.*, 2014; Smith *et al.*, 2018). Several studies have aimed at engineering AAV serotypes with better cell-type and tissue specificities and an improved immune-evasion potential (Gao *et al.*, 2005; Weinmann *et al.*, 2017). AAV9 and AAVrh10 serotypes have been shown to cross the blood–brain barrier and efficiently transduce cells in the CNS, with widespread and sustained transgene expression in the spinal cord and brain even after just a single injection (Borel *et al.*, 2016; Dirren *et al.*, 2015; Thomsen *et al.*, 2014). Importantly, they can efficiently target neurons and astrocytes, making them the most applicable delivery systems for treating ALS.

Several researchers have independently use siRNA or shRNA to silence mutant SOD1 expression in vitro and in vivo (Foust *et al.*, 2013; Miller *et al.*, 2005; Ralph *et al.*, 2005; Raoul *et al.*, 2005). Intramuscular delivery of siRNA targeting mutant SOD1 in SOD1G93A mice delays the onset of motor neuron symptoms and extend their survival (Miller *et al.*, 2005). Similarly, SOD1G93A mice treated with injection of AAV encoding shRNA against human SOD1 mRNA (hSOD1) exhibited delayed diseases onset and significantly increased their survival by 23% (Foust *et al.*, 2013). The same group later demonstrated the efficacy of this approach in SOD1G93A rats, showing that silencing of hSOD1 expression selectively in the motor cortex also delayed disease onset and prolonged survival (Thomsen *et al.*, 2014). Silencing of SOD1 using an artificial miRNA (miR-SOD1) systemically delivered using the viral vector AAVrh10 in SOD1G93A mice was also found to significantly delayed disease onset, preserved muscle motor functions and extended survival (Borel *et al.*, 2016). Interestingly, similar findings were observed in non-human primates treated with AAVrh10-miR-SOD1 (Borel *et al.*, 2016; Wang *et al.*, 2014). These findings suggest that miRNA silencing strategy warrants further investigations and may offer promise for the development for the treatment of SOD1-related ALS.

7.8.2 Antisense oligonucleotides (ASOs)

The concept of ASOs was first introduced in 1978, when Stephenson and Zamecnik used a chemically modified oligonucleotide, designed to bind to its complementary sequence in a Rous sarcoma virus transcript to inhibit its gene expression and viral replication (Stephenson *et al.*, 1978). ASOs are synthetic single-stranded oligonucleotides that activate the RNAse H, an endonuclease in the nucleus, to degrade the complementary mRNA. They can be designed to

specifically target mutant RNAs or mRNA splicing (Bennett *et al.*, 2010). An ASO therapy based (nusinersen) approach designed to promote exon skipping has proven to be very effective in treating spinal muscular atrophy (SMA) in clinical trials (Chiriboga *et al.*, 2016; Finkel *et al.*, 2016; Mendell *et al.*, 2017; Scoto *et al.*, 2017). In late 2016, this antisense drug (marketed as Spinraza) has received FDA approval for the treatment of SMA. This was the first exciting success of ASO therapeutics in neurodegeneration and a significant milestone for ASO therapy, in general. With increased understanding of gain- and loss-of-function mechanisms of genetic forms of ALS, ASOs therapies have also been tested principally tested in SOD1 and C9ORF72 models to target the mutant forms of RNA but not the wild-type.

The first study using an ASO to target SOD1 showed an effective silencing of SOD1 and reduced mutated SOD1 protein throughout the brain and spinal cord of SOD1G93A rats (Smith *et al.*, 2006). Infusion of ASOs complementary to hSOD1 mRNA extended survival in SOD1G93A rats (Smith *et al.*, 2006). Given these promising preclinical results, the ASO IONIS-SOD1Rx (ISIS 333611 and BIIB067) has been proposed as a therapeutic strategy for SOD1-link ALS and has been clinical tested. In a phase I testing, intrathecal administration of the ASO IONIS-SOD1Rx was showed to be both practical and safe in SOD1 ALS patients (Miller *et al.*, 2013). A phase Ib/IIa trial (NCT02623699) is currently underway to further evaluate safety, tolerability, and pharmacokinetics of IONIS-SOD1Rx. Altogether, the preclinical and clinical tests suggest that ASOs delivered to the CNS represent a feasible treatment for SOD1-related ALS and are safe, however, ASOs are not specific for mutant over wild-type SOD1 and the long-term effects of the reduction of SOD1 need further investigation.

In addition, silencing of SOD1 can be induced by exon skipping of hSOD1 using ASOs complementary to splicing regulatory elements on the primary transcript (Biferi *et al.*, 2017). For instance, administrating an exon-2-targeted ASO embedded in a modified U7 small-nuclear RNA and delivered by AAV10, in either newborn or adult (P50) SOD1G93A mice, was shown to increase survival and restore neuromuscular function (Biferi *et al.*, 2017). These recent findings provide new hope for treatment of ALS and open perspectives for a clinical development.

Strong evidence supports that the mechanism by which the GGGGCC repeat expansion in C9orf72 causes the diseases is by toxicity of RNAs that they generate. Thus early development of ASO-based therapeutics for C9orf72 ALS focused on reducing gain-of-function toxicity associated with the repeat expansion. Testing of the efficacy of ASO-based therapeutics for C9orf72 was initially performed on clinically relevant human IPSC-derived neurons and fibroblasts (Donnelly *et al.*, 2013; Lagier-Tourenne *et al.*, 2013; Sareen *et al.*, 2013). More recently, ASOs

were also evaluated in mouse models expressing the expanded C9orf72 (Jiang *et al.*, 2016; O'Rourke *et al.*, 2015).

Antisense oligonucleotides were designed to bind within the GGGGCC repeat expansion or within surrounding N-terminal regions of the C9orf72 mRNA transcript to either degrade the transcript or block the interaction between the repeat expansion and RNA-binding proteins (Donnelly *et al.*, 2013). ASOs effectively reduced RNA foci formation, dipeptide proteins, increased survival from glutamate excitotoxicity and restored normal gene expression markers (Donnelly *et al.*, 2013; Jiang *et al.*, 2016; Lagier-Tourenne *et al.*, 2013; O'Rourke *et al.*, 2015; Sareen *et al.*, 2013). These promising findings suggest that ASO-based therapy can be a powerful way for treating C9orf72 ALS. They also provided the basis for the initiation of the first C9orf72 ASO clinical trial that is anticipated to start by the end of 2018.

These planned ASOs trials in ALS as well as ongoing trials of ASOs in SMA, Huntington's disease and Alzheimer's disease will enhance our understanding of this therapeutic approach. Importantly, positive outcomes from these clinical trials will revolutionize the treatment of genetically mediated neurodegenerative diseases.

7.8.3 Small molecules targeting RNA

RNAs adopt discrete secondary and tertiary structures and have pivotal roles in biology and diseases (Bernat *et al.*, 2015). The ALS-associated C9orf72 GGGGCC repeat RNA can stably fold to into a four-stranded structure formed by the stacking of planar tetrads of four guanosine residues, termed G-quadruplex (Fratta *et al.*, 2012; Huppert, 2008). This G-quadruplex structure can affect various RNA processing including splicing and translation (Simone *et al.*, 2015). In particular, the C9or72 repeat RNA G-quadruplexes have been shown to specifically sequester RNA-binding proteins and have toxic functions (Haeusler *et al.*, 2016). GGGGCC repeat RNA sequence can also adopt a hairpin structure in addition to G-quadruplexes (Haeusler *et al.*, 2014; Su *et al.*, 2014). Hairpin is composed of a base-paired stem and a loop and it can affect transcription and alternative splicing (Kuznetsov *et al.*, 2008). Targeting these RNA structures of the C9or72 repeat is a potential therapeutic strategy.

Recent developments in technologies and approaches have made the long sought-after goal of developing small-molecule drugs that target RNA possible (Bernat *et al.*, 2015; Connelly *et al.*, 2016; Disney, 2013). Small molecules binding to RNA hairpin or G-quadruplex structure have been identified (Di Antonio *et al.*, 2012; Su *et al.*, 2014). This has provided the springboard to initiate the search for small molecules that can specifically target C9orf72 repeat RNA and hinder

pathogenic interactions with RNA-binding proteins and/or by interfering with RAN translation (Simone *et al.*, 2018; Su *et al.*, 2014) (Figure 2C).

Su et al. (2014) showed that (GGGGCC)8 RNA can adopt a hairpin structure in equilibrium with a quadruplex structure. They designed three compounds targeting mainly the hairpin structure of the (GGGGCC)n RNA and showed that the bioactive small molecule 1a significantly inhibited RAN translation and foci formation in cultured cells (GGGGCC)66 repeat expansion and in patient-derived neurons (Su *et al.*, 2014). However, these small molecules were only tested in vitro on cellular models. Recently, a drug screen study to identify compounds that specifically target the C9orf72 RNA G-quadruplex structure led to the identification of three lead compounds (Simone *et al.*, 2018). These compounds were then functionally validated as ALS therapeutics in C9orf72 IPSC-derived neurons and C9orf72 repeat-expressing fruit flies. Interestingly, two of the lead compounds reduced RNA foci formation and the levels of toxic dipeptide repeat proteins in IPSC-derived spinal motor neurons and cortical neurons (Simone *et al.*, 2018). The most effective small molecule (DB1273) was then tested in vivo on C9orf72 repeat-expressing fruit flies and was found to significantly reduce dipeptide repeats levels. Furthermore, D1273 improved the survival of the fruit flies (Simone *et al.*, 2018). These studies support the further development of small molecules that selectively bind GGGGCC RNA as a therapeutic strategy for C9orf72 ALS and FTLD.

7.9 Limitations of RNA-targeted therapeutic strategies

RNA-targeted therapeutic approaches offer a treatment strategy with greater specificity, improved potency, and decreased toxicity compared to the small molecules against traditional drug targets (signaling proteins). They represent an important way to treat ALS and other neurodegenerative diseases that need to be considered in the near future. However, there are still some concerns and challenges to overcome for ALS therapeutic applications.

Off-target effects RNAi and ASO remain an important consideration though thorough toxicological and safety research prior to clinical application can diminish some of this concern. The negative charge of siRNA and ASO as well as their size makes it difficult for them to cross the cell membrane. Viral packing is currently widely used to deliver ASO and siRNA into cells. Although, viral vectors are highly efficient as transfer vehicles, immunogenicity of the viral vectors is a major concern. Various other delivery strategies such as nanoparticles, liposomes and aptamers could be more effective and safe. Efforts are also underway to chemically stabilize siRNA, which will avoid the need for viral vectors (Castanotto *et al.*, 2009). RNA foci and dipeptide products are

generated from both sense and antisense directions of the C9orf72 transcript. However, ASOs for C9orf72 ALS preferentially target sense strand transcripts. There may be a need to design ASO strategies to target toxic RNA transcribed from both directions in order to adequately treat the C9orf72 ALS (Schoch *et al.*, 2017). Furthermore, ASO-based therapeutic strategy for C9orf72 ALS only target gain-of-function mechanisms, but loss-of-function mechanisms may also act in synergy to cause pathogenesis in C9orf72 ALS. It is very plausible that an integrated therapeutic approach to inhibit toxic RNA foci/dipeptide repeat protein formation and restore normal levels of C9orf72 may be necessary to fully address the cellular deficits in C9orf72 ALS.

7.10 Conclusion

TDP-43, SOD1, FUS, and C9orf72 mutations are involved at various aspects of RNA processing and many of which are shared. It is becoming clear that impaired RNA regulation and processing is a central feature ALS pathogenesis. Given that defects at multiple steps of RNA processing impair cellular function and survival, RNA metabolism can be considered an essential target for therapeutic intervention for ALS and other neurodegenerative disease such as FTLD. The application of RNA-based therapies to modulation of gene and subsequent protein expression is an attractive therapeutic strategy. The preclinical testing of RNA-based therapies targeting SOD1 and C9orf72 mutations are indeed very promising. Similar studies are yet to be undertaken for FUS and TDP-43 mutations. RNA-based therapies could be considered in the future for the treatment of ALS.

Gene	Protein encoded	Regulation of RNA processing
SOD1	Superoxide dismutase 1	Yes
TARDBP	Tar-DNA-binding protein-43	Yes
FUS	Fused in sarcoma	Yes
C9orf72	C9orf72	Yes
ATXN2	Ataxin-2	Yes
TAF15	TATA-box binding protein associated factor 15	Yes
UBQLN2	Ubiquilin 2	No
OPTN	Optineurin	No
KIF5A	Kinesin family member 5A	No
hnRNPA1	Heterogeneous nuclear ribonucleoprotein A1	Yes
hnRNPA2 B1	Heterogeneous nuclear ribonucleoprotein A2/B1	Yes
MATR3	Matrin 3	Yes
CHCHD10	Coiled-coil-helix-coiled-coil-helix domain containing 10	No
EWSR1	EWS RNA binding protein 1	Yes
TIA1	TIA1 cytotoxic granule associated RNA binding protein	Yes
SETX	Senataxin	Yes
ANG	Angiogenin	Yes
CCNF	Cyclin F	No
NEK1	NIMA related kinase 1	No
TBK1	TANK binding kinase 1	No
VCP	Valosin containing protein	No
SQSTM1	Sequestosome 1	No
PFN1	Profilin 1	No
TUBB4A	Tubulin beta 4A class IVa	No
CHMP2B	Charged multivesicular body protein 2B	No
SPG11	Spatacsin vesicle trafficking associated	No
ALS2	Alsin Rho guanine nucleotide exchange factor	No

Table 7-1: ALS genes and their involvement in RNA processing.

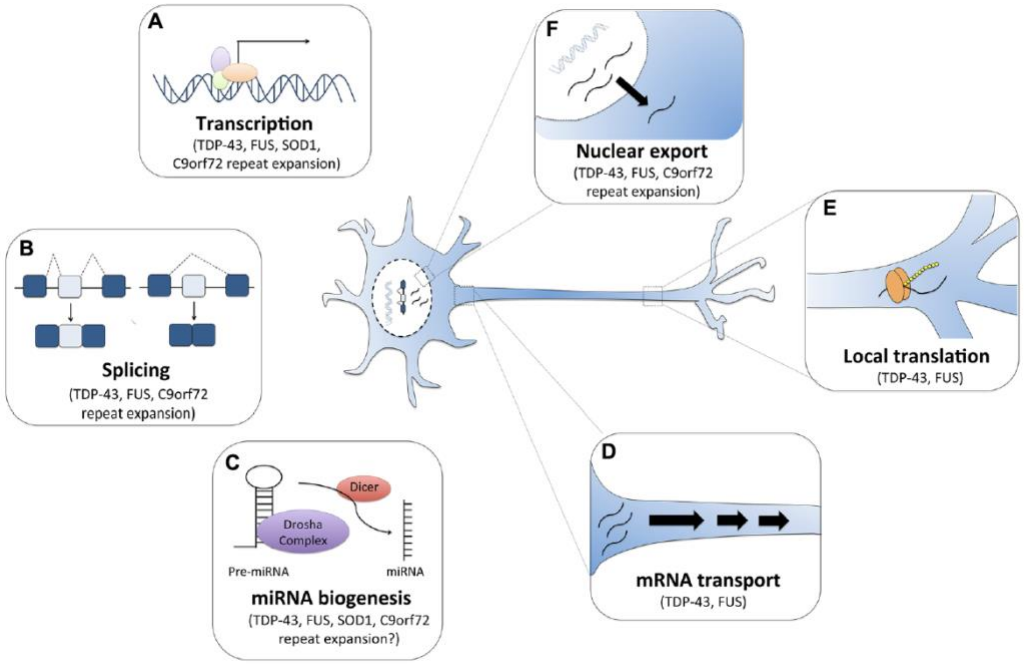

Figure 7-1: RNA dysfunction in amyotrophic lateral sclerosis (ALS).

Major ALS mutations may disrupt RNA processing by several mechanisms. For instance, (A) mutations in ALS genes SOD1, TDP-43, FUS and C9orf72 can alter gene expression. (B) The RNA binding proteins TDP-43 and FUS can affect global splicing machinery. Dipeptide repeat proteins from C9orf72 intronic expansion can also alter splicing patterns of specific RNAs. (C) TDP-43, FUS, and dipeptide proteins can also promote microRNA biogenesis as components of the Drosha and Dicer complexes. TDP-43 and FUS also alter mRNA transport (D) and local translation (E). (F) TDP-43 and FUS predominantly reside in the nucleus, but when mutated they are can mislocalization to the cytoplasm where they bind and regulate different sets of RNAs including the export and mislocalization of other transcripts to the cytoplasm. Poly-PR dipeptide can also bind nuclear pores channels blocking the import and export of molecules.

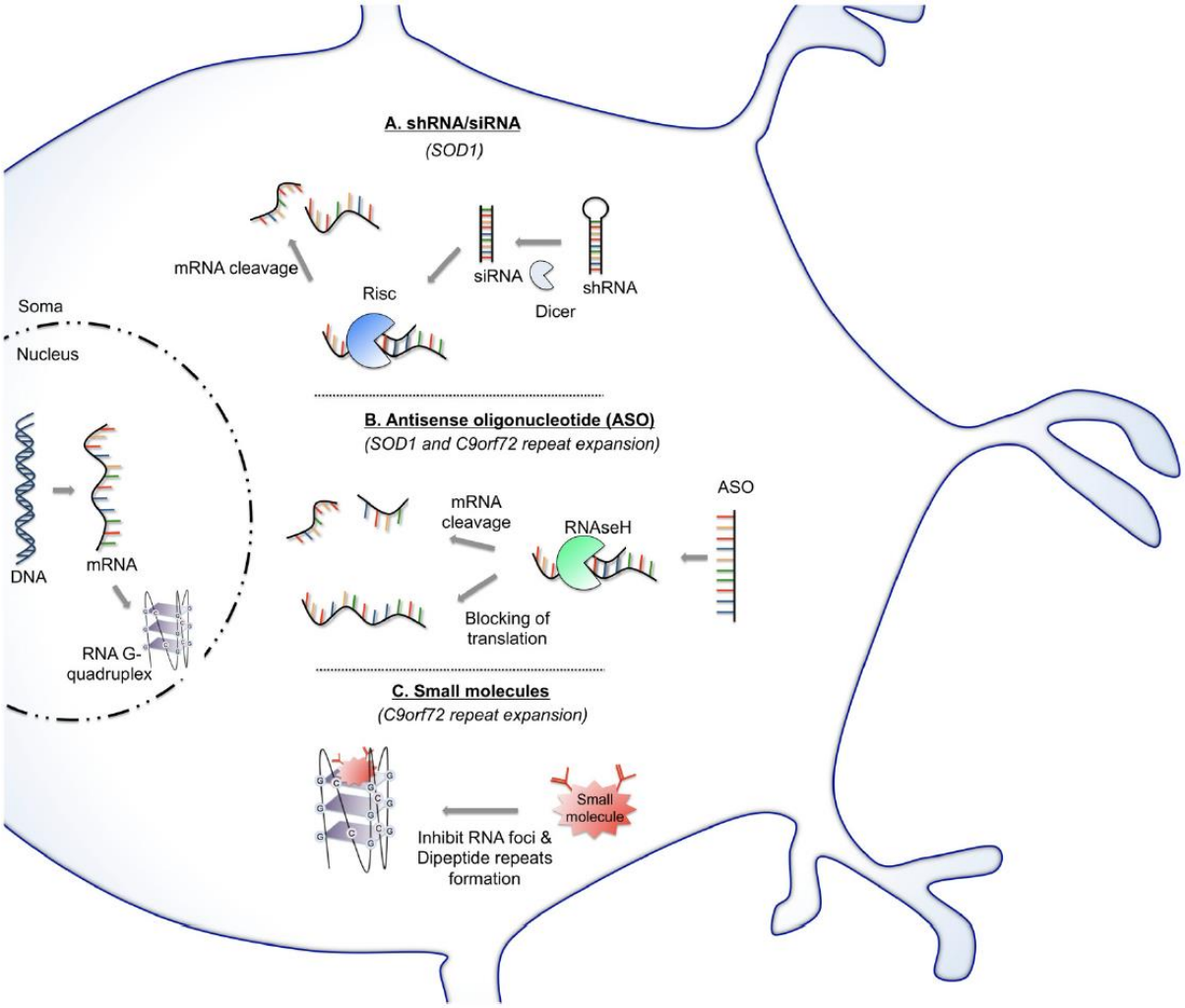

Figure 7-2: RNA-based therapy approaches for potentially treating ALS.

(A) SiRNAs operate through RNA interference pathway. After strand unwinding, one siRNA strand binds argonaute proteins as part of the RNA-induced silencing complex (RISC) and is recruited to a target mRNA which is then cleaved. Virus can provide a means of shRNA, which will be cleaved once in the cytoplasm by dicer enzyme into siRNA. This approach has been evaluated to reduce the level of mutant SOD1 protein. (B) Antisense oligonucleotide (ASO) binds to targeted mRNA and induces its degradation by endogenous RNase H or blocks the mRNA translation. This strategy is being exploited as a potential therapeutic avenue in ALS aiming principally to reduce the protein level of SOD1 protein or by targeting of C9orf72 RNA foci. (C) Small molecules can be designed to target and stabilize RNA structures. This approach was particularly tested to stabilize G-quadruplex of C9orf72 GGGGCC repeat RNA. Stabilization of G-quadruplex structure reduces RNA foci formation and blocks repeat translation.

7.11 Authors contributions

SP contributed to the idea conception and overall review design. ZB and SP wrote the manuscript.

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8 APPENDIX III: MODELLING C9ORF72-RELATED AMYOTROPHIC LATERAL SCLEROSIS IN ZEBRAFISH

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8.1 Abstract

A hexanucleotide repeat expansion within the C9orf72 gene is the most common genetic cause of amyotrophic lateral sclerosis (ALS) and its discovery has revolutionized our understanding of this devastating disease. Model systems are a valuable tool for studying ALS pathobiology and potential therapies. The zebrafish (Danio rerio) has particularly become a useful model organism to study neurological diseases, including ALS, due to high genetic and physiological homology to mammals, and sensitivity to various genetic and pharmacological manipulations. In this review we summarize the zebrafish models that have been used to study the pathology of C9orf72-related ALS. We discuss their value in providing mechanistic insights and their potential use for drug discovery.

8.2 Introduction

Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disease characterized by the degeneration of the upper motor neurons in the motor cortex and the lower motor neurons in the spinal cord. The disease typically begins with a loss of muscle strength and gradually leads to paralysis. Eventually, most patients will die within two to five years after diagnosis due to weakness of the respiratory muscles (Brown *et al.*, 2017; Kiernan *et al.*, 2011). The disease occurs with an estimated incidence of 1:100,000 and a lifetime risk of 1:1000. To date, there is no existing treatment that provides significant clinical benefits. Only Riluzole and edaravone are currently approved and can prolong patient survival to a very limited degree (Abe *et al.*, 2014; Bensimon *et al.*, 1994). Most cases of ALS are sporadic (sALS), but about 10% are familial (fALS) and have a strong inherited link (Pasinelli *et al.*, 2006).

In 1993, SOD1 (Cu-Zn superoxide dismutase 1) was the first gene to be associated with fALS (Rosen *et al.*, 1993). Over the past two decades, several mutations in more than two-dozen genes have been discovered to in ALS, including TARDBP (transactive response to DNA binding protein 43kDa), FUS (fused in sarcoma) (Kwiatkowski *et al.*, 2009; Sreedharan *et al.*, 2008; Vance *et al.*, 2009) and C9orf72 (C9orf72-SMCR8 complex subunit) (DeJesus-Hernandez *et al.*, 2011; Renton *et al.*, 2011). Importantly, an abnormal GGGGCC repeat expansion in the first intron of the C9orf72 gene is the most common genetic cause in ALS patients (DeJesus-Hernandez *et al.*, 2011; Renton *et al.*, 2011). About 40% of patients with the familial form of ALS and 8–10% of sALS patients have the C9orf72 hexanucleotide repeat expansion (Majounie *et al.*, 2012). The hexanucleotide repeat expansion in the C9orf72 gene is also associated with frontotemporal dementia (FTD). Some reports have suggested that ALS patients harbour a higher number of

repeats than FTD patients (Dols-Icardo *et al.*, 2014; Suh *et al.*, 2015). Nevertheless, how a repeat expansion in the C9orf72 gene causes neurodegeneration in both ALS and FTD is still poorly understood. Since the discovery of C9orf72 mutation, several animal models including the worm (Caenorhabditis elegans), the drosophila, the mouse and the zebrafish have been used to investigate the C9orf72 form of ALS (Ciura *et al.*, 2013; Julien *et al.*, 2006; Mizielinska *et al.*, 2014; Therrien *et al.*, 2013).

With about 70% homology between human and zebrafish genes, this animal model appears to be a powerful organism for studying the C9orf72 form of ALS in a vertebrate (Howe *et al.*, 2013). Zebrafish as a model is easily genetically manipulated, thus ideal for creating stable transgenic lines to investigate loss- or gain-of-function phenotypes Shaw, 2018 #456}(Schmid *et al.*, 2013a). Moreover, the translucency of this model allows simple visualization of the organs/systems of the embryo and to some extent at adult stage in zebrafish casper mutant. In the last few years, the zebrafish has also proven itself for high-throughput drug screening and confirm the capacity of a molecule to rescue neuronal and motility phenotypes, including in ALS models (Patten *et al.*, 2017). The use of this vertebrate model may thus o er an exciting opportunity to identify new therapies for the C9orf72 form of ALS.

This review summarizes what is currently known about the pathogenesis of ALS associated with the C9orf72 gene. An overview of the use of zebrafish as a model system is provided and the different zebrafish models that have been developed over past couple years to study the pathological mechanisms of ALS related to the C9orf72 gene are then discussed.

8.3 The C9orf72 Gene associated with ALS

The human C9orf72 gene consists of 11 exons and, by alternative splicing, encodes 3 variant transcripts, of which the first and third are translated into the same isoform (481 amino acids) and the second variant is translated into a shorter protein (222 amino acids) (DeJesus-Hernandez *et al.*, 2011). Its sequence is highly conserved between different species including common model systems such as mouse (98.1%) and zebrafish (76.0%). An abnormal GGGGCC repeat expansion in the first intron of the C9orf72 gene is the most common genetic cause in ALS patients (DeJesus-Hernandez *et al.*, 2011; Renton *et al.*, 2011). The GGGGCC hexanucleotide repeat expansions reside between exon 1a and 1b of the gene. The majority of healthy individuals have 11 hexanucleotide repeats in the C9orf72 gene while hundred to several thousand repeats have been reported in ALS/FTD heterozygous carriers (Rutherford *et al.*, 2012; van Blitterswijk *et al.*, 2012). The pathological expansion repeat-length threshold has not been clearly established

but an arbitrary cut-off carrying 30 repeat-alleles is used in most studies. In the last few years, many developments have occurred to explain how the GGGGCC repeat expansion in the C9orf72 gene is associated with ALS. There are currently 3 proposed pathogenic mechanisms by which the repeat expansions, both sense and antisense (GGGGCC)n, cause ALS. These involves two toxic gain-of-function mechanisms, such as RNA foci accumulation (Zu *et al.*, 2013) and protein toxicity by aberrant dipeptide repeat protein accumulation (Ash *et al.*, 2013; Mori *et al.*, 2013a). Alternatively, repeat expansions cause a decrease in C9orf72 mRNA and protein expression, suggesting loss-of-function by C9orf72 haploinsufficiency may also contribute to C9orf72 ALS. Reports have shown that abnormal repeat expansion within the C9orf72 causes haploinsufficiency due to its interference with gene transcription (Ciura *et al.*, 2013; DeJesus-Hernandez *et al.*, 2011). In addition, cytosine hypermethylation of CG dinucleotides in CpG islands (regions of DNA with significantly higher frequency of CG sequence that regulate transcription at their associated promoters) is an important epigenetic modification that could lead to gene silencing (Xi *et al.*, 2013). Indeed, several studies have observed decreased levels of C9orf72 mRNA and protein in C9orf72 patients CNS tissues, lymphoblastoid cell lines and neurons derived from patient-derived induced pluripotent stem cells (IPSCs) (Belzil *et al.*, 2013; DeJesus-Hernandez *et al.*, 2011; Frick *et al.*, 2018). To date, the function of the C9orf72 protein still remains unclear, but it has been reported to contain a DENN domain (differentially expressed in normal and neoplasia). The proteins of this family are highly conserved and are GDP/GTP exchange factors (GEF) that activate Rab-GTPases, suggesting that C9orf72 is involved in GTPase activity and the regulation of vesicular transport (Levine *et al.*, 2013). Indeed, recent studies have reported that the C9orf72 protein function in a complex with the WDR41 and SMCR proteins as a GEF for Rab8 and Rab39 (Corbier *et al.*, 2017; Sullivan *et al.*, 2016). C9orf72 has also been proposed to play a role in autophagic flux (Sullivan *et al.*, 2016) (Ugolino *et al.*, 2016; Yang *et al.*, 2016), endosomal trafficking (Sellier *et al.*, 2016; Shi *et al.*, 2018; Zhang *et al.*, 2018c) and regulating AMPA receptor levels (Xiao *et al.*, 2019). For example, the SQSTM1 protein (p62) is an autophagy protein and knockdown of C9orf72 in human cell lines showed an accumulation of cytoplasmic aggregates of p62, reflecting an impairment in the autophagosome-lysosome system in these cells (Sellier *et al.*, 2016; Webster *et al.*, 2016). This observation is consistent with p62-positive inclusion bodies found in ALS and FTD cases (Al-Sarraj *et al.*, 2011). Additionally, knockdown of C9orf72 in human cell lines have shown to result in cytoplasmic aggregates of the RNA binding protein, TDP-43 (Sellier *et al.*, 2016; Webster *et al.*, 2016). C9orf72 can interact directly with Importin-β1 or Ran-GTPase (Xiao *et al.*, 2015) and may play a role in nucleocytoplasmic transport (Jovicic *et al.*, 2016; Zhang *et al.*, 2015). Consequently, defects in

this pathway may in part account for the mislocalization of TDP-43 from the nucleus to cytoplasm and TDP-43 pathology in ALS. One of the gain-of-function mechanism hypotheses is that the transcription of the repeat expansion in sense and antisense direction leads to the accumulation of RNA foci (Gendron *et al.*, 2013). It has been reported in previous studies that patients with the C9orf72 form of ALS have an accumulation of RNA foci in the brain and the spinal cord (Cooper-Knock *et al.*, 2015; DeJesus-Hernandez *et al.*, 2017; DeJesus-Hernandez *et al.*, 2011; Gendron *et al.*, 2013; Lagier-Tourenne *et al.*, 2013; Mizielinska *et al.*, 2013; Zu *et al.*, 2013). The RNA foci recruit the RNA-binding proteins, inducing their mislocalization and consequently preventing them from performing their normal function, leading to altered RNA metabolism (Gitler *et al.*, 2016). For example, Lee et al. in 2013 (Lee *et al.*, 2013) showed that the hnRNP-H protein binds directly to GGGGCC RNA and colocalizes with RNA foci. A list of proteins that bind RNA foci has been identified from previous in vivo and in vitro studies (Haeusler *et al.*, 2016). The other toxic gain-of-function mechanism is the formation of dipeptide repeat proteins (DPRs) due to associated non-ATG (RAN) translation of transcripts in the sense and antisense directions. There are five di erent DPRs, Gly-Ala (poly-GA), Gly-Arg (poly-GR), Gly-Pro (poly GP), Pro-Ala (poly-PA) and Pro-Arg (poly-PR), forming insoluble aggregates in the nucleus and cytoplasm of the cells (Ash *et al.*, 2013; Mori *et al.*, 2013a; Zu *et al.*, 2013). Dipeptides containing arginine appear to be particularly toxic to cells, they tend to bind the nucleolus and disrupt RNA splicing and ribosomal biosynthesis (Wen *et al.*, 2014). For example, research has shown that poly-GR colocalize with ribosomal subunits, thus affecting translation (Zhang *et al.*, 2018d). Poly-PR, on the other side, seem to bind the nuclear pores and block the nuclear transport of RNA and proteins (Freibaum *et al.*, 2015). The relative contribution of these 3 mechanisms remains to be established as well as the molecular pathways leading to motor neuron degeneration. It is however becoming clearer that haploinsufficiency might contribute to the disease process in synergy with the gain-of-toxicity mechanism (Zhu *et al.*, 2020). Animal models can then provide answers on the different mechanisms involved and consequently help to develop a specific treatment for patients with C9orf72 ALS.

8.4 Zebrafish as a model system

Zebrafish as a vertebrate model has received favorable attention from clinicians owing to its many advantages for the study of disease. The rapid, external development of translucent embryos together with low-cost husbandry has sparked the establishment of several models of human diseases in zebrafish (Schmid *et al.*, 2013a), and these models often closely resemble the human condition (Lieschke *et al.*, 2007). Additionally, the zebrafish genome has been sequenced (Howe

et al., 2013) and more than 80% of zebrafish genes have a high degree of conserved gene structures across vertebrate species as well as 50–80% amino acid identity with most human homologs, including homologs for over 70% of disease-causing genes. The zebrafish are also especially powerful for genetic manipulation. The majority of their genes can be manipulated by gain and loss-of-function approaches and consequently makes the zebrafish model system particularly valuable for the study of genetic diseases such as ALS.

The easiest and first approach used to study loss-of-function mutations in zebrafish is the injection of antisense morpholino oligonucleotides (MO) to transiently block translation or splicing (Phillips *et al.*, 2014). This method only allows the phenotype to be observable over a short period of time and can also leads to off-target toxicity (Bedell *et al.*, 2011; Kok *et al.*, 2015). This is why this technique is more useful for drug testing at the embryonic or larval stages (Bedell *et al.*, 2011). As for the study of gain-of-function mutations, mRNA injection also provides a transient mutation and consequently presents the same limitation as the injection of antisense morpholino oligonucleotides, i.e., the impossibility to study late-onset phenotypes (Bedell *et al.*, 2011). The Tol2 transposon system is also popular for integrating a DNA sequence into the zebrafish genome, but it is often necessary to out-crossing over several generations to obtain the desired stable transgenic line (Kawakami *et al.*, 2000; Lissouba *et al.*, 2018; Suster *et al.*, 2009). However, the development of stable transgenic models does have some limitations, such as ectopic expression, toxic overexpression and variability due to genetic background (Kabashi *et al.*, 2011b). Genomic engineering tools, including site-specific transgenesis such as Cre-loxP and the Gal4/UAS system, are an exciting approach for genetic model of ALS in zebrafish (Halpern *et al.*, 2008; Lin *et al.*, 2013). New genome-editing techniques, including transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats (CRISPR), have been developed in the last few years and significantly facilitate the study of loss- or gain-of-function mutations in zebrafish (Hisano *et al.*, 2014; Hwang *et al.*, 2013). It is now possible to rapidly generate a zebrafish line with the wanted mutation that will be inherited in order to study its phenotype.

The zebrafish has emerged as a very attractive model for the study of neurological diseases. The basic structure of central nervous system in zebrafish has all the major domains found in the mammalian brain and they produce the same neurotransmitters such as glutamate, GABA, serotonin, dopamine, histamine and acetylcholine (Babin *et al.*, 2014; Panula *et al.*, 2006). Despite some notable differences in the size of the zebrafish brain, the overall cognitive processing and sensory and retinotectal pathways, share an overall homology with humans

(Tropepe *et al.*, 2003). Noteworthily, the hippocampus is essential for spatial memory in mammals and is derived developmentally from the medial pallium of the telencephalon (Eichenbaum *et al.*, 1999). In zebrafish, unlike mammals, the telencephalon is everted and the lateral pallium appears to be structurally homologous to the mammalian hippocampus (Rodriguez *et al.*, 2002). Importantly, many of the genes implicated in human neurodegenerative diseases, such as ALS, have been identified in zebrafish. The zebrafish motor system has also some similarities to humans (Madgwick *et al.*, 2015; Stil *et al.*, 2016); making it relevant model for motor neuron diseases(McWhorter *et al.*, 2003; Wood *et al.*, 2006). However, an important difference between zebrafish and human is that there is no direct telencephalic projections to the spinal cord (i.e., corticospinal tract) in fish (Babin *et al.*, 2014). The zebrafish spinal cord shows a comparable organization to human spinal cord with groups of motor neurons located in specific regions of the spinal cord and innervating specific muscle fibers. Particularly, zebrafish consist of two classes of motoneurons, primary motor neurons and second motor neurons, based on their formation time and target musculature (Myers *et al.*, 1986). Primary motor neurons are localized relatively dorsally with large cell bodies and thick axons and they innervate the dorsal, middle, and ventral trunk musculature. On the other hand, secondary motor neurons are located more ventrally in the motor column with smaller cell bodies and thinner axons than primary motor neurons and they innervate the dorsal and ventral musculatures. The zebrafish secondary motor neurons are comparable to human alpha motor neurons and innervate ventral as well as dorsal muscle fibers. Of note, the zebrafish primary motor neurons do not have an equivalent in human and likewise, the human gamma motor neurons are not present in zebrafish. The gamma motor neurons are found in species that have limbs or limb-like structures since it is involved in proprioception (Babin *et al.*, 2014).

Several ALS-causing genes have been studied in zebrafish using loss- or gain-of-function approaches. For instance, overexpression of mutant but not wild-type human SOD1 in zebrafish leads to short motor axons with premature branching associated with deficient swimming in response to touch (Lemmens *et al.*, 2007). Expression upon mRNA injection of human TARDBP mRNA containing one of three missense mutations (TARDBPG348C, TARDBPA315T or TARDBPA382T), but not wild type TARDBP at the same level of expression, resulted in motor behavioural defects and hyperbranched ventral root projections to trunk musculature (Kabashi *et al.*, 2010). Injection of human mutant FUS mRNA resulted in disrupted nuclear import (Dormann *et al.*, 2010) and accumulation in cytosolic stress granules (Bosco *et al.*, 2010) and either mRNA or MO injection resulted in locomotor deficits and ventral root projection abnormalities (Kabashi

et al., 2011a). Since the discovery of C9orf72 mutation as the most common genetic cause of ALS, it has received much attention and has also evidently been modelled in zebrafish.

Zebrafish has only 1 C9orf72 orthologue (zgc:100846; c13h9orf72) and similarly to human C9orf72 protein-coding transcripts, the zebrafish also possesses 3 protein-coding transcripts giving rise to 2 protein isoforms if different size (Ciura *et al.*, 2013). Here, we review different C9orf72 zebrafish models (Table 1) that have provided new insights into the pathogenesis of ALS since the abnormal repeat expansion within the C9orf72 gene was identified in 2011 (DeJesus-Hernandez *et al.*, 2011; Renton *et al.*, 2011).

8.5 C9orf72 zebrafish models

8.5.1 Loss of function zebrafish models

A zebrafish MO-based model of c9orf72 was the first in vivo model to support the loss-of-function hypothesis (Ciura *et al.*, 2013). The knockdown model was generated using a MO to block the translation of the protein. MO knockdown of c9orf72 in zebrafish resulted in motor neuron axonopathy, a disturbed arborization and shortened axons at early developmental stages. It also led to motor deficits, in particular, abnormalities in spontaneous and evoked swimming were observed in the knockdown model. Importantly, these phenotypes could be rescued with the expression of human C9orf72 mRNA, confirming that they were due to the direct effects of loss-of-function of c9orf72. This zebrafish knockdown model exhibited important motor behavioural defects as observed in the disease, allowing us to think that the loss-of-function has a contribution in ALS pathogenesis, but is not exclusive. Of note, no major morphological defects and no experiments examining cytoplasmic aggregation of TDP-43 were reported in this zebrafish knockdown model.

Subsequently, Yeh et al. (Yeh *et al.*, 2018) developed in 2018 two constructs in which two portions of the functional domain DENN of c9orf72 were deleted to study its function and to confirm the specificity of the loss-of-function. One of the constructs consisted of only the upstream part of the domain (c9orf72u-DENN) and the other contained the central region (c9orf72c-DENN). The models showed an altered neuronal network and inhibition of the response to stimuli. This work demonstrated that the DENN domain is essential to the function of c9orf72 and that a truncated domain has a dominant negative effect. These models were also able to confirm that a deficiency of c9orf72 affects axon formation in the hindbrain and spinal motor activity. In addition, an increase in the number of neuronal cells in apoptosis was noted. The models showed significantly reduced

GTPase activity compared to controls, which is necessary for vesicular formation and transport. These results suggest the importance of c9orf72 for crucial neuronal functions and synapse formation. They reported that the knockdown of Tp53 was able to rescue the apoptosis phenotype but did not completely restore the motility deficit. They then concluded that c9orf72 regulates neuronal apoptosis through Tp53. The study by Yeh et al. (Yeh *et al.*, 2018) also established a deletion construct of Cyclin G1 (an N-terminal deletion) (ccng1D1-40), and when injected into c9orf72-deficient embryos, it rescued neuronal apoptosis, axonal deficits and motility. This finding suggests that Cyclin G1 upregulation is involved in the pathogenesis of c9orf72-deficient embryos. Thus, the C9orf72-Cyclin G1-Tp53 cascade has an important role in the mechanism behind the disease. Importantly, the zebrafish model with the GTPase activity domain deleted from the C9orf72 protein is sufficient to produce a neurological phenotype similar to the symptoms of ALS patients.

To better understand the role of C9orf72 loss-of-function in ALS pathogenesis, our group recently generated a stable zebrafish line with a reduced expression of c9orf72 (Butti *et al.*, 2021) using a microRNA-based gene-silencing approach developed for zebrafish (Giacomotto *et al.*, 2015). Unlike MO-based knockdown approach, transgenic zebrafish lines that stably express microRNAs designed to target knockdown desired genes of interest have no apparent non-specific toxic effects (Leong *et al.*, 2012). Reduced c9orf72 function in zebrafish resulted in motor defects, muscle atrophy, motor neuron loss and mortality in early larval and adult stages (Butti *et al.*, 2021). TDP-43 form aggregates in neurons, glial cells (Neumann *et al.*, 2006) and axial skeletal muscle (Mori *et al.*, 2019) and this TDP-43 pathology is a hallmark of ALS. Using a specific antibody that recognizes the highly homologous human TDP-43 ortholog in zebrafish (Schmid *et al.*, 2013b), we importantly observed a mislocalization of TDP-43 from the nucleus to the cytoplasm in skeletal muscles of the c9orf72 loss-of-function model; consistent with TDP-43 pathology in ALS. Analysis of the structure and function of the neuromuscular junctions (NMJs) revealed a significant reduction in the number of presynaptic and postsynaptic structures and an impaired release of quantal synaptic vesicles at the NMJ in the c9orf72 loss-of-function model. Furthermore, a strong downregulation of the synaptic protein, SV2a, as well as marked reduction in the number and size of Rab3a-positive synaptic puncta at NMJ were observed upon reduced c9orf72 function in zebrafish, resulting in a reduced rate of synaptic vesicle cycling at the NMJ. Altogether, findings from this zebrafish model suggest that loss-of-function mechanisms may underlie defects in synaptic function at NMJ in ALS.

8.5.2 Gain of function zebrafish models

Hexanucleotide repeats

Soon after the discovery of C9orf72 mutation, Lee et al. (2013) (Lee *et al.*, 2013) used the zebrafish to determine whether expanded GGGGCC transcripts might be toxic and sequester RNA binding proteins in vivo. They particularly injected EGFP constructs containing 8, 38 or 72 repeats into zebrafish embryos to test the length-dependance GGGGCC toxicity. The construct was then expressed only in small portions of the embryos and in a mosaic distribution. While the injection of 8xGGGGCC DNA vector did not increase the number of cells in apoptosis at 24h post-fertilization, injection of 38 and 72 GGGGCC DNA vectors in zebrafish led to a significant increase in the number of apoptotic cells. Noteworthy, zebrafish injected with 72 GGGGCC repeats displayed a higher rate of apoptosis compared to those injected with 38 GGGGCC repeats. However, RNA foci were only detected in embryos injected with 72 repeats. These embryos were also caspase-3-positive, with clear nuclear condensation and fragmentation, and supporting cell death due to injection of long repeat expansions. This study was thus able to demonstrate in vivo using the zebrafish model that the expression of longer repeats produces RNA foci that initiate cell apoptosis.

The gain-of-function zebrafish C9orf72 model developed by Swinnen et al. in 2018 (Swinnen *et al.*, 2018) complements previous studies by showing that overexpression of 35, 70 or 90 GGGGCC repeats led to motor axon abnormalities but not overexpression of ten or less repeats. These motor axon defects consisted primarily of reduced axonal growth and aberrant branching at 30 h post-fertilization. This study also reported for the first time that the same deficit was noticed with the injection of 70 hexanucleotide antisense repeat RNAs but injection of 35 GGGGCC repeats was less toxic. Importantly, RNA foci were observed in zebrafish injected with 90 sense and 70 antisense repeats. To further test whether gain-of-function mechanism relies on RNA toxicity or toxic DPR, or both, they also generated 50 repeats of DPRs containing an ATG start codon. Interestingly, zebrafish expressing DPRs containing arginine, specifically GR and PR, exhibited motor axon abnormalities. However, their analyses indicated that repeat RNA toxicity was independent of DPR toxicity. Since RNA foci are known to bind and sequester RNA-binding proteins, Swinnen and colleagues further took advantage of their model to investigate whether different RNA binding proteins such the Pur-alpha protein (Cooper-Knock *et al.*, 2015; Gitler *et al.*, 2016) were also able to bind the toxic RNA repeats, as well as the involvement of p62, a protein involved in autophagy (Almeida *et al.*, 2013). Expression of Pur-alpha inhibited the development of motor axonal defects induced by sense repeat RNA in zebrafish. It also

decreased RNA foci formation and increased the levels of p62 protein. The study demonstrated that, through its modulating effect on p62, Pur-alpha prevented axonopathy induced by repeat RNAs in zebrafish. Importantly, the use of zebrafish as model system provided insights in the occurrence of RNA toxicity independently of DPR toxicity in the pathogenesis of the C9orf72 form of ALS.

A stable C9orf72 transgenic zebrafish model expressing the hexanucleotide repeat expansion was recently developed and characterized by Shaw and colleagues in 2018 (Shaw *et al.*, 2018). A DNA construct consisting of 89 hexanucleotide repeats under the ubiquitin promotor was injected into the zebrafish embryos to generate the C9orf72 transgenic. Two viable lines were generated (2.2-2 zebrafish line and 2.2-7 zebrafish line) and both exhibited an accumulation of RNA foci and DPRs in muscle and central nervous system (CNS). DPRs were formed by conventional ATG-dependent translation and RAN translation, and in sense and antisense directions. Further characterization of the two 2.2 lines revealed that 2.2-7 zebrafish had a marked reduction in survival rate at 15 days postfertilization and started to exhibit motor behavioural defects as of 5 days postfertilization. Behavioural (swimming) deficits worsened during adulthood and the fish had reduced body weight, consistent with phenotypes observed in ALS. Additionally, muscle atrophy, loss of motor neurons, cognitive impairment, and early mortality in young adults as observed in patients with the C9orf72 form of the disease were also noted in the zebrafish model. They also reported that the heat shock response (HSR) was activated in the model, as found in ALS patients, and this activation correlated with the disease progression (Miyazaki *et al.*, 2016). This stable C9orf72 transgenic zebrafish model presented the most pathological hallmarks of the ALS and it appeared to be a powerful organism for drug screening.

Dipeptide repeat proteins (DRP)

Zebrafish transgenic UAS responder lines with an ATG codon forcing the translation of the poly-GA protein (GA80-GFP) or without an ATG codon (ggggcc80-GFP) were generated by Ohki et al. in 2017 (Ohki *et al.*, 2017) using the Tol2 transposon system. They were interested in establishing a model expressing poly-GA, since this DPR was found in higher quantities in the brain of patients with C9orf72 repeat expansions (Mori *et al.*, 2013a; Schludi *et al.*, 2015). First of all, both lines presented a pathological hallmark of C9ORF72 ALS, consisting of the presence of RNA foci in neurons within the spinal cord. The expression of ggggcc80-GFP had a mild toxicity in zebrafish while the expression of GA80-GFP was highly toxic. Indeed, these fish displayed severe pericardial edema, reduced circulation of red blood cells, and aggregates of GA80-GFP

exclusively in the muscles. On the other hand, zebrafish expressing GA80-GFP did not have significant differences in axon length and did not show any vascular patterning defects. However, the endothelial cells in this model were found to be thinner and less well-structured due to a lack of blood perfusion. Ohki and colleagues also injected a MO to specifically targets poly-GA translation, and not the repeat expansion, which this resulted in the rescue of the severe pericardial edema phenotype. This model was used to confirm that poly-GA is highly toxic in zebrafish and suggests the possibility of targeting DPRs as a therapeutic strategy.

After determining that GR was the most toxic of the DPRs, and that 200 GR repeats were largely sufficient to cause significant developmental and motor deficits, Swaminathan et al. (Swaminathan *et al.*, 2018) developed in 2018 a stable transgenic zebrafish model expressing GR repeats associated with C9orf72. They overexpressed 100 GR repeats under the control of the upstream activation sequence (UAS) using the Tol2 transposon system. In the construct, a GFP sequence under the cmlc2 promoter was used to allow easy identification of the transgenic embryos. Gal4 driver lines under three different promoters were crossed with this transgenic line for cell-specific expression of GR repeats-Hsp: Gal4 for ubiquitous expression after a heat shock, mnx: Gal4 for motor neuron expression, and elavl3: Gal4 for neuronal expression. Ubiquitous expression of GR in zebrafish resulted in severe morphological and motor deficits. On the other hand, GR expression only in motor neurons, led to significant motors deficits with no gross morphological abnormalities. In addition, a decreased in motor neuron length, and an increased in apoptosis cells in the spinal cord were observed in zebrafish expressing GR specifically in motor neurons. However, GR repeat expression did not seem to affect the development of motor neurons. In this study, the zebrafish DPR model demonstrated that GR repeat expression is sufficient to induce motor dysfunction and shortening of motor neuron length in a similar manner to what is observed in ALS patients.

Cytoplasmic accumulation of TDP-43 is a significant neuropathological hallmark in C9-ALS patients. There is indeed a clear association between TDP-43 accumulation and degeneration as well as clinical phenotype (Mackenzie *et al.*, 2013). However, none of these gain-of-function zebrafish studies described above reported the presence of cytoplasmic TDP-43 in modelling C9orf72-ALS. Additionally, in these zebrafish models the levels of the C9orf72 protein were not examined. It would have been relevant to check whether expressing the abnormal expansion repeat or dipeptide repeat proteins (DPRs) in zebrafish has an impact on C9orf72 protein levels since some studies have shown a decrease in brain of ALS patients (Waite *et al.*, 2014).

8.6 Conclusion

ALS is a fatal motor neuron disease and there is an urgent need to develop and assess more effective therapeutics. The GGGGCC hexanucleotide repeat expansion in the C9orf72 gene is the most common mutation in both familial and sporadic cases of ALS. The generation of C9orf72 zebrafish models have provided novel insights into the pathogenesis of C9-ALS. It is, however, important to point out that the signature GGGGCC hexanucleotide repeat expansion motif in the C9orf72 is only present in human and its closest relative, the chimpanzee (Iyer *et al.*, 2018). Although, the expression of pathogenic GGGGCC repeat expansions in zebrafish result in RNA foci and DPR formation that are toxicity as in ALS, perhaps the best approach to accurately model C9orf72-related ALS in zebrafish, is to attempt knock-in the repeats in intron 1 of the zebrafish c9orf72 with CRISPR genome editing approaches in the future.

In addition to providing a better comprehension of why the hexanucleotide repeat expansion in the C9orfF72 gene is pathogenic, the zebrafish has the advantage of being a powerful organism for the screening of therapeutic compounds compared to other animal models. The zebrafish C9orf72 model in the study by Shaw et al. in 2018 (Shaw *et al.*, 2018) is a successful example of its use for the discovery of new drugs in a short period of time. They were able to confirm that Ivermectin, a compound that was effective in their SOD1 zebrafish screen, also decreases HSR activation in the C9 zebrafish model, as well as Riluzole (McGown *et al.*, 2016; Shaw *et al.*, 2018). Zebrafish are consequently a powerful tool for drug-discovery by allowing rapid drug screening. The prospect of zebrafish for drug discovery opens new avenues using zebrafish ALS models for finding treatments.

Study	Mechanism	Stable or Transient	Method(s)	Cellular and Behavioral Phenotype
Ciura et al., (2013) [18]	Loss of function	Transient	Morpholino knockdown C9orf72	• Axonopathy (disturbed arborization and shortened axons of motor neuron axons) • Moto deficits (abnormalities of spontaneous and evoked swimming)
Lee et al., (2013) [51]	Gain of function	Transient	Overexpression (38× and 72× G_4C_2)	• Increase of apoptotic cells throughout 24 h embryo • RNA foci formation with 72× G_4C_2
Ohki et al., (2017) [84]	Gain of function	Stable	UAS responder line expressing 80 GGGGCC without an ATG codon (gggggcc80-GFP) was crossed to the Gal4 driver line SAGFF73A for ubiquitous expression	• RNA foci formation • A mild cardiac phenotype
	Gain of function	Stable	UAS responder line expressing 80 GGGGCC with an ATG codon (GA80-GFP) was crossed to the Gal4 driver line SAGFF73A for ubiquitous expression	• RNA foci formation • A severe cardiac phenotype • A reduced circulation of red blood cells • Aggregates of GA80-GFP found in musculature
Swinnen et al., (2018) [85]	Gain of function	Transient	mRNA Overexpression (35×, 70× and 90× G_4C_2 and C_2G_4)	• Axonopathy (disturbed arborization and shortened axons of motor neuron axons) • RNA foci formation
Swaminathan et al., (2018) [86]	Gain of function	Stable	UAS transgenic line overexpressing 100 GR repeats was crossed to Gal4 driver lines under three different promoters: Hsp: Gal4 for ubiquitous expression after a heat shock, mnx1: Gal4 for motor neuron expression, and elavl3: Gal4 for neuronal expression.	• Reduction in motor neuron length • Increase apoptotic cells in the spinal cord • No effect on motor neuron development • An impaired motor functions • Reduction in swimming behaviour • Respond poorly to touch
Yeh et al., (2018) [87]	Loss of function	Transient	Overexpression of 2 deletion variants, one containing only de upstream DENN domain and one containing only the central DENN domain (c9orf72$^{\alpha\text{-DENN}}$ and c9orf72$^{\text{c-DENN}}$)	• Altered neuronal network • Axon formation in the hindbrain altered • Response to stimuli is inhibited • Spinal motor activity altered • Increase of apoptotic cells • Reduced of GTPase activity
Shaw et al., (2018) [21]	Gain of function	Stable	Zebrafish model expresses 89 C9*orf72* hexanucleotide repeat expansions	• RNA foci formation in muscles • Antisense and sense DPRs in muscles and CNS • Altered swimming behaviour • Reduced weight gain • Mortality • Muscle atrophy • Motor neuron loss • Heat shock response is activated

Table 8-1: Summary of the different zebrafish C9orf72 models and their distinct phenotypes.

8.7 Author Contributions

Conceptualization, S.A.P.; writing—original draft preparation, G.F.; writing—review and editing, S.A.P. and Z.B.; visualization, G.F., Z.B. and S.A.P.; supervision, S.A.P.; project administration, S.A.P.; funding acquisition, S.A.P. All authors have read and agreed to the published version of the manuscript.

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