

# **Modelling Amyotrophic Lateral Sclerosis in Zebrafish: An Integrated Analysis Focusing on Chemically Induced Models**

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Master in Bioengineering – Molecular Bioengineering

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## Resumo

A esclerose lateral amiotrófica (ELA) – a doença mais comum dos neurónios motores – é uma condição neurodegenerativa que causa uma perda progressiva de função motora. Os doentes sofrem de sintomas motores e não-motores que, eventualmente, impossibilitam a realização de funções básicas; a morte ocorre por falência respiratória, quando os músculos da respiração deixam de funcionar. Dado que as terapias existentes conseguem apenas atrasar moderadamente a progressão da doença, existe uma necessidade de novas opções terapêuticas para a ELA. O peixe-zebra é um pequeno vertebrado com um grande potencial como animal modelo para investigação fundamental e desenvolvimento de fármacos, tendo vantagens únicas face a abordagens *in vitro* e a modelos em mamíferos. Nas últimas décadas, o interesse em criar modelos de ELA em peixe-zebra tem crescido, o que contribuiu para a atual riqueza de ferramentas e modelos disponíveis. Contudo, as descrições dos modelos existentes de ELA em peixe-zebra são altamente heterogêneas, e a informação sobre ferramentas e ensaios relevantes permanece dispersa. Adicionalmente, apesar da maioria dos casos de ELA ter causa desconhecida (ELA esporádica, idiopática), os esforços de criação de modelos têm-se focado na modificação genética de alvos que justificam uma pequena proporção de casos; exceções notáveis incluem propostas para o uso da exposição a bisfenol A (BPA) ou  $\beta$ -metilamino-L-alanina (BMAA) como modelo de ELA esporádica. Nesta dissertação, revimos a literatura sobre os modelos de ELA em peixe-zebra, ferramentas de geração de modelos e ensaios relevantes para a ELA, incluindo estudos recentes que abordam combinações de toxinas e alvos genéticos emergentes. Discutimos a sua validade e identificámos oportunidades de investigação na área. Para além disso, analisámos especificamente a validade dos modelos de ELA esporádica induzidos por BPA e BMAA, ao estender a sua caracterização fenotípica – incluindo uma avaliação multiparamétrica da atividade, sono, axónios motores e, para o modelo induzido por BPA, stresse oxidativo e proteostasia. A validade preditiva da exposição a BPA para o desenvolvimento de fármacos para a ELA também foi avaliada através de experiências de resgate dos fenótipos motores induzidos por BPA, com fármacos aprovados para uso em doentes com ELA. Descrevemos hipoatividade dependente da concentração de BPA (afetando a atividade de enrolamento da cauda em embriões com um dia de idade e a natação espontânea em larvas com 4 a 6 dias de idade) e de BMAA (afetando apenas os enrolamentos da cauda); os efeitos do BPA e do BMAA no comprimento e morfologia dos axónios motores; aumento no dano

oxidativo e alterações mitocondriais em larvas expostas a BPA; e resultados promissores no resgate com edaravona, um tratamento aprovado para a ELA, de fenótipos induzidos por BPA. Globalmente, esta dissertação evidencia a pertinência, oportunidades e limitações dos modelos de ELA em peixe-zebra, e suporta a validade dos modelos de ELA esporádica induzidos quimicamente, para aplicações de investigação específicas.

# Abstract

Amyotrophic Lateral Sclerosis (ALS), the most common motor neuron disease, is a neurodegenerative condition that causes a progressive loss of motor function. Patients suffer from motor and non-motor symptoms, eventually becoming unable to perform basic functions. Death comes from respiratory failure when the breathing muscles fail. Since existing therapies can only modestly delay the progression of the disease, there is a need for new ALS treatments. Zebrafish is a small vertebrate with great potential as a model animal for fundamental research and drug development, with unique advantages over both *in vitro* approaches and mammal models. In the last decades, there has been a growing interest in modelling ALS in zebrafish, which contributed to the current wealth of available tools and models. However, the descriptions of zebrafish models of ALS are highly heterogeneous, and information on relevant tools and assays remains disperse. Additionally, while most ALS cases have unknown causes (sporadic, idiopathic ALS), modelling efforts have been focused on genetic modification of targets that explain a small proportion of cases; notable exceptions include proposals for bisphenol A (BPA) or  $\beta$ -methylamino-L-alanine (BMAA) exposure as a sporadic ALS model. In this dissertation, we reviewed the literature on existing zebrafish models of ALS, model generation tools, and ALS-relevant assays, including recent studies that focus on emerging toxin combinations and gene targets. We discussed their validity and identified research opportunities in the field. Furthermore, we specifically analysed the validity of the BPA- and BMAA-induced models of sporadic ALS, by extending their phenotypic characterisation – including a multi-parameter assessment of activity, sleep, motor axons and, for the BPA-induced model, oxidative stress and proteostasis. The predictive validity of BPA exposure for ALS drug development was also evaluated in rescue experiments of induced motor phenotypes with ALS drugs approved for human use. We describe the concentration-dependent hypoactivity caused by BPA (affecting tail coiling activity in 1-day-old embryos and spontaneous swimming in 4 to 6-day-old larval fish) and BMAA (affecting only the tail coiling activity); effects of BPA and BMAA on the length and morphology of motor axons; increased oxidative damage and mitochondrial alterations in BPA-exposed larvae; and promising results on the rescue of BPA-induced phenotypes with edaravone, an approved treatment for ALS. Globally, this dissertation evidences the pertinence, opportunities, and limitations of ALS modelling in zebrafish, and supports the validity of chemically induced models of sporadic ALS for specific research applications.



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# Abbreviations

4-HNE: 4-hydroxynonenal

ALS: Amyotrophic lateral sclerosis

ALSFRS-R: Revised ALS functional rating score

ALS-PDC: ALS-parkinsonism-dementia complex

AMO: Antisense morpholino oligonucleotide

ANOVA: Analysis of variance

AO: Acridine orange

BMAA:  $\beta$ -methylamino-L-alanine

BPA: Bisphenol A

CRISPR: Clustered regularly interspaced palindromic repeats

DNA: Deoxyribonucleic acid

dpf: Days post-fertilisation

DPR: Dipeptide repeat

ENU: N-ethyl-N-nitrosourea

ER: Endoplasmic reticulum

fALS: Familial ALS

FDA: Food and Drug Administration

FTD: Frontotemporal dementia

FUS: Fused in sarcoma

G4C2: GGGGCC hexanucleotide

GRP75: Glucose-regulated protein 75 kDa

GRP78: Glucose-regulated protein 78 kDa

HERV-K: Human endogenous retrovirus K

hpf: Hours post-fertilisation

HSP70: Heat shock protein 70 kDa

LAM: Locomotor activity monitor(ing)

MCLR: Microcystin leucine and arginine

miRNA: Micro RNA

mRNA: Messenger RNA

NMJ: Neuromuscular junction

NPH: Nikon-Prior-Hamamatsu

PBS: Phosphate-buffered saline

PBST: PBS with 0.05% Tween

PVDF: Polyvinylidene difluoride

RNA: Ribonucleic acid

sALS: sporadic ALS

SDHA: Succinate dehydrogenase complex subunit A

SDS-PAGE: Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

SEM: Standard error of the mean

sgRNA: Single guide RNA

SOD1: Superoxide dismutase [Cu-Zn]

TALEN: Transcription activator-like effector nucleases

TARDBP / TDP-43: Transactive response DNA binding protein 43 kDa

TILLING: Targeting induced local lesions in genomes

TUNEL: Terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labelling



# Acknowledgements

This thesis is the culmination of a five-year journey through the (previously Integrated) Master in Bioengineering, one that shaped me as an engineer, scientist, and human being, and for which I will forever be grateful. There are some people without whom this journey and, specifically, this project and thesis would not have been possible, and who I would like to thank:

Professor Jorge Ascensão Oliveira, my supervisor, for his scientific, intellectual, and professional guidance. I am deeply thankful for his critical assistance in the conceptualisation, execution, and revision of this work; his warm welcome to the team; the autonomy and opportunities that I was granted; the mutual trust and respect that were present in every interaction; the interests and curiosities that we shared; and his deep insights into the world, science, and the subjects of this thesis.

Doctor Brígida Pinho, my co-supervisor, who was always available and willing to guide me through many all-important decisions in experiment planning and analysis; who shared her knowledge and expertise on a vast array of experimental techniques; who gave me often-critical motivation and fresh perspectives; and who shared with Prof. Jorge Oliveira the task of supervising the execution of this project.

The entire Mitoneuro team, particularly Ângela Oliveira and Liliana Almeida, who made me feel at home in the lab, shared countless helpful tips and interesting conversations, and made the whole lab run smoothly; Rui Silva, who laid groundwork for this project through his contributions to locomotor activity analysis; Francesca Scaini, Ilaria Pometcu, Leonor Lima, and Marcela Bota, some of the youngest students that passed through the group, lab mates at all times and helpful hands in many moments.

The technicians, Maria do Céu Pereira and Mónica Caldas, who played a critical role in ensuring the availability and conservation of lab materials, while always greeting us with genuine warmth.

The professors and teachers of the (Integrated) Master in Bioengineering, who gave me the knowledge, tools and inspiration to understand complex themes, continuously grow personally and professionally, and get out of my comfort zone.

All my friends, especially Ana Miranda, André Oliveira, André Silva, Bruno Moraes, Carlos Paiva, Catarina Lopes, Diogo Moutinho, Inês Vilela, João Magalhães, Maria Loureiro, Rafael Santos, Raquel Pereira, and Ricardo Briosso – for years, we shared joys

and pains, worked, travelled, played, sang, danced, and so much more. I always felt like I belonged, even when some coursework or contagious disease forced us all to stay home.

My parents, Nuno Oliveira and Paula Santos, who made me the person I am and gave me the opportunity to do everything I did. Together with my brother, Daniel Oliveira, they are my home and my roots, and I am deeply thankful for the love and trust that we share.

Finally, my girlfriend and best friend, Ana Beatriz Silva, whose steadfast support, love and companionship make all things easier and happier; teach me so much about the world, life, and myself; allow me to grow past self-imposed boundaries; and inspire me to always be the best version of myself.

## Funding

This work was developed in UCIBIO/REQUIMTE, Department of Drug Sciences, Faculty of Pharmacy of the University of Porto, with financial support from Fundação para a Ciência e Tecnologia (FCT): PTDC/CTA-AMB/31544/2017, LA/P/0140/2020 (i4HB), UIDP/04378/2020, UIDB/04378/2020, 2022.04458.PTDC.



# Outcomes

## Oral communications

Oliveira NAS, Silva RFO, Pinho BR, Oliveira JMA (2022, May 4–6) Extending the Phenotypic Characterization of Zebrafish Models of Parkinsonism and Amyotrophic Lateral Sclerosis. *Encontro de Investigação Jovem da Universidade do Porto [IJUP] – 15 edição*, Porto, Portugal.

Oliveira NAS, Silva RFO, Pinho BR, Oliveira JMA (2022, May 5) Motor Activity and Sleep Phenotypes in a Zebrafish Model of Sporadic Amyotrophic Lateral Sclerosis. *I Meeting of the Disease Pathways and Biomarkers Group @UCIBIO*, Porto, Portugal.

## Manuscripts in preparation

Oliveira NAS, Pinho BR, Oliveira JMA. Modelling Amyotrophic Lateral Sclerosis in Zebrafish [Manuscript in preparation] (review).

Oliveira NAS, Pinho BR, Oliveira JMA. Behavioural and Molecular Analysis of Chemically Induced Zebrafish Models of Amyotrophic Lateral Sclerosis [Manuscript in preparation] (experimental).





# 1. Introduction and Objectives

## 1.1. Introduction

“Morrie had amyotrophic lateral sclerosis (ALS), Lou Gehrig's disease, a brutal, unforgiving illness of the neurological system. There was no known cure.” [2]

ALS is a neurodegenerative disease that primarily affects the motor neurons, usually leading to death after 3–5 years, during which patients progressively lose the ability to use their muscles [3, 4]. ALS imposes a major burden on patients and healthcare systems [5], and current therapies can only modestly modify disease progression [3]. ALS cases are often categorised as familial (fALS) or sporadic (sALS), depending on the presence of disease-causative hereditary genetic mutations. Most cases are sporadic and with unknown causes [3, 6]. A combination of genetic and environmental factors is likely involved in the aetiology of ALS [3, 7].

The development of new and better therapies for ALS requires candidate drugs to undergo extensive preclinical and clinical testing. The discovery and preclinical stages of drug development are based on cells, tissues, and animal models – systems that aim to mimic some relevant aspects of healthy humans or patients [8]. Zebrafish (*Danio rerio*) is an increasingly popular animal for this purpose [9, 10]. Its advantageous features include high experimental tractability [9-14], throughput [10], and biological relevance [14-17].

The use of zebrafish in ALS studies has been growing in the last decades (Fig. 1), and currently there is a wide variety of proposed models and assays for drug discovery and development [11, 18], which would benefit from being systematised and analysed from

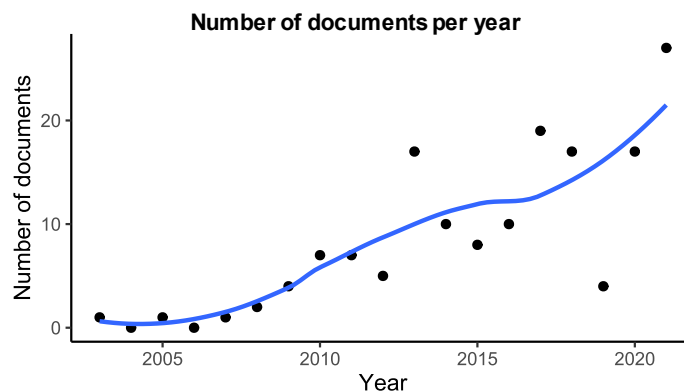


**Fig. 1 – Zebrafish is an increasingly popular animal to study ALS.** Number of documents in the Scopus database that contain “zebrafish” and “amyotrophic lateral sclerosis” in the title, abstract, or keywords, per year of publication, from 2003 to 2021.

an integrated perspective. Past efforts to model ALS in zebrafish have mostly focused on genetic modification strategies, effectively modelling the subset of ALS patients with a known genetic cause [11]. Nevertheless, chemically induced (namely through bisphenol A or  $\beta$ -methylamino-L-alanine exposure) models of sporadic ALS in zebrafish have been proposed and partially described [19-23], though further research is needed to establish their validity and applicability, prior to widespread adoption.

### 1.2. Objectives

In this dissertation, we aimed to:

- Integrate and review the state-of-the-art knowledge on the use of zebrafish to model ALS, by reviewing the literature on model generation, phenotypes, and previously described models.
- Characterise the face validity of chemically induced models of sporadic ALS in zebrafish, specifically regarding their behaviour, motor neurons, and molecular parameters.
- Characterise the predictive validity of chemically induced models of sporadic ALS, by evaluating the rescue of their behavioural phenotypes by FDA-approved ALS drugs.

### 1.3. Thesis outline

Besides this introductory chapter, this dissertation includes four more main chapters:

- Chapter 2 is a literature review of zebrafish models of ALS. It presents the features of *Danio rerio* as a relevant model animal, existing tools for model generation, ALS-relevant phenotypes that can be evaluated in zebrafish, established and emerging zebrafish models of ALS. Chapter 2 also identifies research opportunities in the area.
- Chapter 3 is the original research section. In it, chemically induced models of sporadic ALS are characterised regarding their face validity (including behavioural phenotypes, motor neuron morphology and molecular parameters) and predictive validity (through rescue with FDA-approved ALS drugs).
- Chapter 4 discusses the main findings from chapters 2 and 3, integrating them and answering the proposed objectives of the dissertation.
- Chapter 5 is the list of cited references in the entire dissertation.





## 2. Literature Review

### Modelling Amyotrophic Lateral Sclerosis in Zebrafish

#### Abstract

Amyotrophic Lateral Sclerosis (ALS) is a neurodegenerative disease that leads to progressive disability and motor impairment, posing a major burden to global health. The existing therapies provide only modest improvements in patient survival, so there is an important need to develop new treatments for ALS. Zebrafish is an extremely promising model animal for translational drug development for ALS – it is an experimentally tractable vertebrate, with a high homology to humans and an ample experimental toolbox. The last decade has seen an increased interest in ALS modelling in zebrafish, leading to the current abundance and variety of described methods and models. Additionally, the rise of gene editing techniques and toxin combination studies has created novel opportunities for ALS studies in zebrafish. In this review, we address the relevance of zebrafish as a model animal for ALS studies, the methods for model generation and relevant phenotypical evaluation, and the established and emerging zebrafish models of ALS. We critically analyse the validity of these models, including their value for drug development, and discuss potential research opportunities in this area.

## **2.1. Introduction to amyotrophic lateral sclerosis**

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder primarily characterised by the degeneration of upper and lower motor neurons, but which also affects frontotemporal and other brain neurons [6]. This neurodegeneration leads to motor and non-motor symptoms, which invariably progress to death, usually from respiratory failure [3, 6]. ALS is the most common motor neuron disease, with an incidence of 0.3 to 3.3/100.000 person-year [24]. This corresponds to a relatively high lifetime risk of developing the disease – that risk has been estimated at 1 in 347 for men and 1 in 436 for women in a European population [25]. The only approved treatment in Europe is riluzole, which prolongs survival by an average of 3 months [26]. In 2017, the FDA approved the use of edaravone as a treatment for ALS in the United States, in addition to riluzole. Edaravone slows disease progression (33% smaller decline in the revised ALS functional rating score over 24 weeks, versus patients receiving placebo) in patients with recent and mild symptoms [27, 28].

Approximately half of the patients diagnosed with ALS present cognitive or behavioural impairment, and 13% of cases develop concomitant frontotemporal dementia (FTD) [29]. ALS and FTD also share associated sleep disorders, including excessive daytime sleepiness and increased sleep latency and fragmentation [30-34]. Besides these overlapping clinical presentations, ALS and FTD have similar genetic and pathophysiological signatures; therefore, they are sometimes considered parts of the same spectrum of disease [35-37].

About 5–20% of ALS cases have a hereditary genetic cause – familial ALS (fALS) – but the remaining cases still have an unclear aetiology – sporadic ALS (sALS). Despite this classification, ALS is highly heterogeneous, and its aetiology likely involves both genetic and environmental factors [3, 7]. The most frequently mutated genes are *C9ORF72*, *SOD1*, *TARDBP*, and *FUS* [38], but many other mutations have been associated with ALS [3, 39]. Additionally, environmental risk factors for ALS include smoking [40], military deployment [41], and exposure to heavy metals and pesticides [42]. Exposure to  $\beta$ -methylamino-L-alanine (BMAA), a non-proteinogenic amino acid produced in some cyanobacterial blooms, is also linked to ALS and ALS-parkinsonism-dementia complex (ALS-PDC) [43-45]. Lastly, an increased expression of human endogenous retrovirus K (HERV-K) in ALS patients suggests a possible role of its viral

particles [46, 47]. However, the epidemiological relevance of many of these risk factors is still unclear [42, 48, 49].

At the cellular level, several mechanisms are implicated in motor neuron degeneration in ALS, including loss of proteostasis leading to protein inclusions, altered RNA metabolism and stress granule dynamics [37], mitochondrial dysfunction, excitotoxicity, oxidative stress, impaired nucleocytoplasmic transport, impaired axonal transport and cytoskeletal dynamics, altered vesicle transport and inefficient DNA repair [3, 39, 50]. Externally to the motor neuron, neuroinflammation (microgliosis and astrogliosis) and oligodendrocyte dysfunction also contribute to ALS. These processes culminate in the loss of axonal projections and neuromuscular junctions (NMJ), leading to neuronal death and muscle denervation [3].

The study of animal models is essential to understand the pathological mechanisms of ALS and to develop new treatments for it, given its heterogeneity, complexity, and unclear aetiology. For instance, the mutant SOD1 (G93A) mouse is one of the earliest and most studied ALS models [51], which contributed to the discovery of the applicability of edaravone to ALS [52]. More models have since been described, based on genetic manipulation of *SOD1*, *TARDBP*, *FUS*, and *C9ORF72*, as well as other genes and chemical exposures in different species: *C. elegans*, fruit flies, zebrafish, mice, rats and non-human primates [53]. Small animal models make high-throughput trials possible, whereas assays with mammalian models have higher physiological relevance [54]. In that respect, zebrafish strikes a balance as a relevant intermediate between mammals and simpler animals, since it is a vertebrate with fast development, high fecundity and tractability.

In this review, we aggregate relevant information for researchers planning an ALS study in zebrafish. We focus on the features of zebrafish models of ALS, the available toolbox for their generation, and relevant behavioural, biochemical, electrophysiological, and imaging assays. We critically describe well established and emerging models, their development, and their utility for future ALS studies and drug testing, identifying research opportunities.

### 2.2. Zebrafish as a disease model

#### 2.2.1. Zebrafish biology and features as an ALS model

Zebrafish (*Danio rerio*) is a small tropical fish from southeast Asia, that was initially used as a model organism for developmental studies [9]. More recently, it has been established as a prominent disease model for drug screening and preclinical trials [9, 10].

Several advantageous features, relative to its biology and experimental tractability, explain the emergence of zebrafish as a model organism. Regarding biological advantages, zebrafish are vertebrates and have a high degree of conservation of genes and processes implicated in human neurodegenerative disorders, including ALS [14-17]. Regarding tractability, zebrafish are much cheaper to breed and house than mammalian models [11]. Additionally, *in vivo* and whole-organism microscopy techniques are facilitated by zebrafish's *ex vivo* fertilization and optically transparent larvae [10]. This makes the imaging of motor neurons and ALS-relevant pathology possible [14]. The speed of zebrafish development, together with a high fecundity, are also extremely advantageous for high-throughput screening approaches [10]. Other favourable features of *D. rerio* include its stereotyped movement patterns and responses, which allow the sensitive detection of ALS-related motor deficits [11-13]; and its large genetic toolbox – including knockdown, overexpression, knockout, transgenesis, and gene editing tools [9, 10, 14] – which has been extensively used to generate ALS models, as will be detailed in the next section.

Naturally, zebrafish models also have some important caveats, including their larger phylogenetic distance from humans than mice or other mammals [55]. Additionally, research studies primarily use larval and embryonic stages, but that limits the possibility of observing adult-onset phenotypes [14]. Thus, most models must show an accelerated form of disease. Such fast-developing phenotypes are also common in other animal models of human disease; they increase tractability at the cost of construct validity. Importantly, an additional whole-genome duplication event took place in the zebrafish evolutionary history, when compared to mammals. This event led to the existence of many ohnologous genes (<http://ohnologs.curie.fr/>) in the zebrafish genome, and to consequent subfunctionalization and neofunctionalization phenomena. Therefore, care must be taken when targeting specific genes, by searching for ohnologues and unexpected functions [10, 56]. Lastly, zebrafish lack a direct neural connection between the telencephalon and the spinal cord. In fact, no zebrafish structure is analogous to the

human corticospinal tract – the pathway that connects the motor cortex, in the telencephalon, to the spinal neurons [57]. Thus, the degeneration of corticospinal upper motor neurons that happens in ALS is not modelled in zebrafish [14].

Altogether, the advantageous features of zebrafish explain why it is increasingly used for ALS modelling and drug discovery, since its fast reproduction and development expedite the testing of a large number of candidate therapeutic molecules [10]. Zebrafish is a highly tractable model animal and a promising ALS research tool, which can bridge important gaps between *in vitro* and mammalian models.

### 2.2.2. Toolbox for model creation

Zebrafish models of ALS can be induced by chemical or genetic means. This section describes different model induction methods, from a methodological and technological standpoint. Chemical induction is generally achieved by exposing embryos to the relevant molecule through the incubation medium [20-23, 58, 59]. It is crucial to select an appropriate concentration range and control for solvent effects, so that the specific disease phenotypes are replicated while minimizing unspecific toxicity. To develop genetic models of ALS in zebrafish, scientists interfere with the function of genes that are implicated in human ALS, leading to their loss of function or gain of (toxic) function.

Transient overexpression of a gene, either wild-type or mutant, can be induced through mRNA microinjection [60-65]. In this procedure, 1 to 3 nanolitres of mRNA solution are injected in the embryo yolk sac, during the 1 to 4-cell stage, through a controlled pressure pulse. Cytosol injections are also possible in the 1-cell stage, granting better efficiency at the cost of higher technical difficulty [66].

Temporary gene expression reduction (knockdown) can be achieved through the microinjection of targeted antisense morpholino oligonucleotides (AMO), similarly to how mRNA is injected for overexpression [61, 63, 65]. However, the use of AMO for loss-of-function experiments has been declining in the last decade: the emergence of genome editing technologies, coupled with the realization that most AMO-induced phenotypes are not present in the respective null mutants – where the gene product is completely absent or non-functional [67] – have put into question many of these models [68, 69]. Nonetheless, if carefully validated (by comparing the AMO phenotype with that of the respective null mutant and rescuing it through mRNA-induced overexpression [70]), AMO models may still be useful, thanks to their comparatively easier generation.

Stable expression of mutant genes can be achieved through mutagenesis strategies, like TILLING [71] and other mutagenesis screens [72]), or through the creation of transgenics [73, 74]. In mutagenesis strategies, adult fish are treated with a mutagen (such as N-ethyl-N-nitrosourea; ENU) prior to breeding. The resulting progeny, harbouring numerous random point mutations, is then screened to select animals with a mutation in the desired gene. Those individuals are backcrossed with wild-type animals to minimize off-target mutations; nevertheless, some mutations in other loci are likely to still be present [71, 75].

Transgenesis allows the expression of any gene, mutant or not, under the regulation of a specific promoter. This has many advantages, including the ability to generate conditional phenotypes by using inducible promoters. However, the random insertion of the transgene may lead to variable off-target effects and expression levels; additionally, false therapeutic effects may be observed in the presence of an inhibitor of the transgene expression [59]. For instance, molecules that inhibit the heat shock response can reduce the expression of transgenes that use a heat shock-inducible promoter, while not modulating any disease-specific pathway [76]. Transgenic zebrafish are commonly generated by the microinjection of 1 to 4-cell embryos with a DNA construct containing the target gene(s), followed by backcrossing with wild-type animals [73-75, 77, 78]. Retroviral vector infection has also been proposed for the insertion of transgenic constructs [79]. The design of DNA or retroviral transgene constructs is a complex task, which has been previously described [79-81].

Lastly, targeted genome editing methods have been developed for zebrafish, using zinc-finger nucleases [82], TALENs [83, 84] or CRISPR-Cas9 [85, 86]. These allow for the stable loss of expression of a gene (knockout), as well as for the insertion of target sequences in a well-defined locus (knock-in) [59, 86]. CRISPR-Cas9 is the most recent of the genome editing systems, efficiently targeting specific genes through the customisation of a synthetic single guide RNA (sgRNA). The sgRNA directs the endonuclease Cas9 to the position in the genome where it induces a double-strand DNA break [85]. Altogether, genome editing tools allow for unprecedented control over genetic modifications in zebrafish, which makes them highly promising for the development of specific and selective ALS models.

### 2.2.3. ALS-related phenotypes and their evaluation

Since the signs and symptoms of ALS cannot be completely mimicked and evaluated in an animal model, biomedical and preclinical research describes and evaluates surrogate phenotypes. This is done through behavioural, biochemical, electrophysiological, and microscopic assays. Fig. 2 illustrates a selection of assays for phenotypical characterisation of ALS models.

The simplest **behavioural** assays evaluate spontaneous motor activity in embryos, in the form of tail-coiling at 24 hours post-fertilization (hpf) [65] or of the swimming pattern and distance travelled by zebrafish larvae [87]. These methods typically use the videorecording of embryos or larvae over a length of time and quantify their locomotor activity, with differences from control indicating motor phenotypes [65]. Spontaneous activity and sleep patterns can also be quantified in 4 to 7 days post-fertilization (dpf) zebrafish using an infrared-based activity monitor, to identify motor phenotypes and circadian rhythm disruptions [88].

The touch evoked escape response assay evaluates reflex responses in 2 to 6 dpf larvae, by touching the head and/or tail with a blunt instrument [22, 65, 89]. The touches are repeated with a consistent time interval, and the number of animals that respond by immediately swimming (or the number of touches that elicited a response for each larva) are recorded. Other reflex response assays include acoustic [58] and visual [88] startle trials, which quantify the locomotor activity of zebrafish larvae following a sudden stimulus. Since reflex deficiencies can be caused by either sensory or motor deficits (and only the latter are relevant for ALS modelling), reflex response assays should be complemented by motor-specific endpoints, such as spontaneous locomotion.

Adult zebrafish memory and learning, which may be relevant in the context of ALS with cognitive decline or FTD, can be evaluated with the T-maze test. This test assesses the time taken by a fish to find and memorise the location of food in a T-shaped opaque tank [90]. The spatial distribution of adult fish in their tank can also be used to assess fear, anxiety and aggression, in diverse experimental setups. In these trials, fish are filmed for a length of time and the time spent in each predetermined tank region is quantified [71, 91].

The swimming endurance test is another ALS-relevant assay, that identifies motor dysfunction in adult fish by monitoring their ability to swim against an accelerating water flow [73]. A modification of the swimming endurance test, where fish have to swim in a

beaker with a spinning current, can also be used [20]. These methods can find motor phenotypes that are too subtle or delayed for detection by larval or embryonal tests.

Regarding **biochemical** assays, the quantification of ALS-associated proteins has been used to verify genetic phenotypes and gather insights on implicated cellular pathways [71]. SDS-PAGE or native gel separation of zebrafish embryo lysis extracts, followed by western blotting, is the most common method used to that end. Dot blots are another useful tool for protein detection when molecular weight separation is not necessary. To specifically evaluate protein aggregation, the extract may be previously filtered through a cellulose acetate membrane with an appropriate pore size. The target protein is then

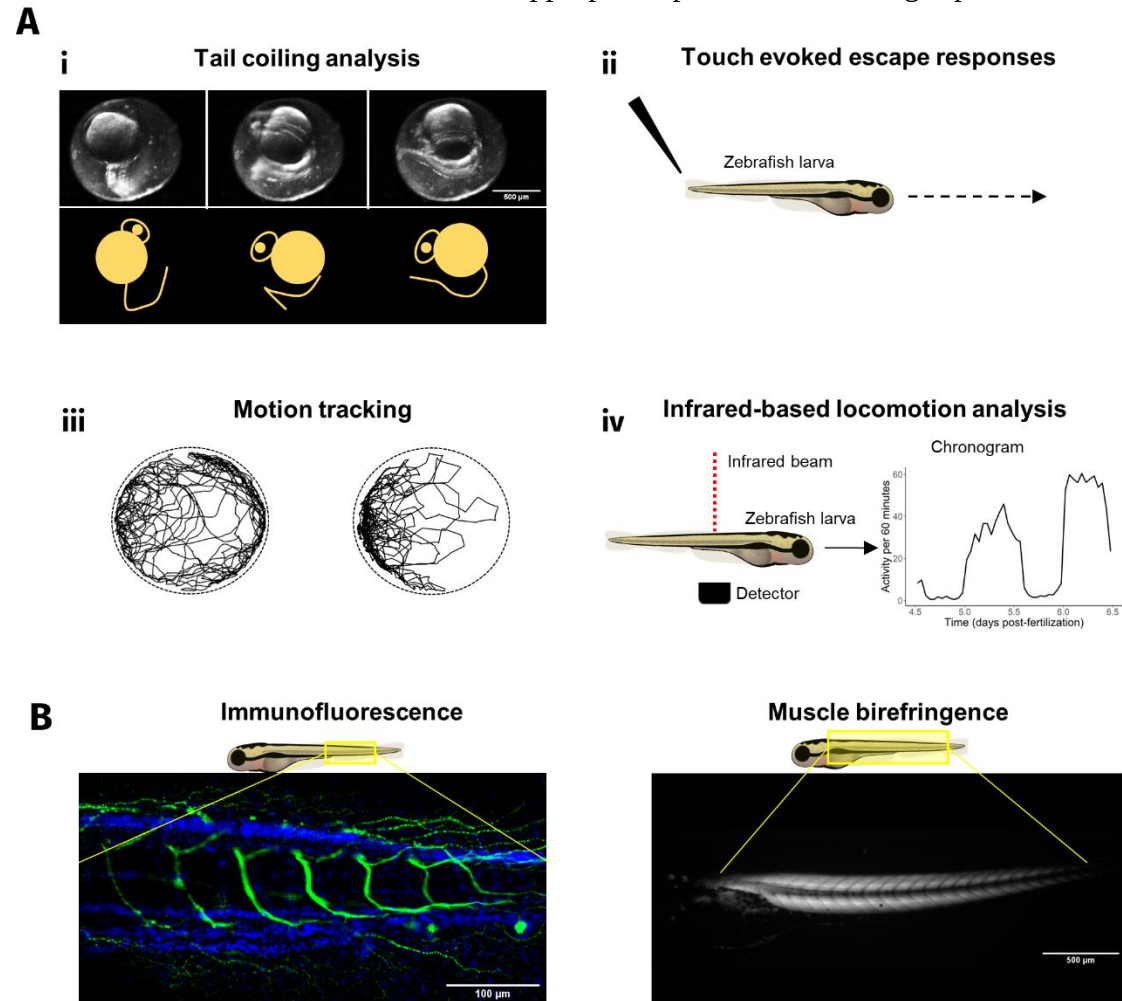


**Fig. 2 – Common methods of phenotypical characterisation of ALS models in zebrafish.** (A) Behavioural endpoints that can identify ALS-consistent phenotypes: (i) embryo tail coiling movements, whose frequency can be altered by neurodegeneration; scale bar, 500 µm; (ii) touch-evoked escape responses, in which larvae are touched in the tail or head and their escape reflex is evaluated; (iii) spontaneous motion of larvae in a well, whose patterns (left – homogeneous swimming pattern, right – heterogeneous swimming pattern), distances, and speed can be assessed; and (iv) spontaneous motion of larvae, assessed by infrared monitoring (Chronogram – plot of activity vs. time of a 4.5–6.5 dpf control zebrafish larva). (B) Microscopy methods for ALS model characterisation: (left) immunofluorescence can be used to identify and evaluate motor axons, here labelled in green in a 5 dpf larva, with nuclei stained in blue; scale bar, 100 µm; (right) muscle birefringence can be observed by polarisation microscopy and used to assess muscle organisation, here visible in an 8 dpf larva; scale bar, 500 µm. Original zebrafish larva drawing by Lizzy Griffiths [1]; all other images and data are from the authors' laboratory.

quantified in the aggregates retained on the filter [92, 93]. Increased oxidative damage and susceptibility to oxidative stress are other pathological hallmarks of ALS that can be evaluated in zebrafish models. For instance, increased embryo mortality after exposure to 5 mM H<sub>2</sub>O<sub>2</sub> indicates increased susceptibility to oxidative stress [71], while redox markers can be measured using mass spectroscopy techniques [94]. Additionally, mass- and nuclear magnetic resonance spectroscopy are important tools for the metabolic characterisation of zebrafish models – they can be used to analyse multiple metabolites and elucidate ALS-associated disease pathways [21].

**Electrophysiological** assays can give information on neuromuscular dysfunction. For instance, miniature endplate currents are small depolarization events that happen in muscle fibres when a unit package of acetylcholine is released from presynaptic terminals at resting potential [95]. These events can be measured in zebrafish larvae by voltage clamping single muscle fibres at the resting potential and recording current fluctuations. Attenuated and slowed miniature endplate currents indicate impaired synaptic transmission at the NMJ [87]. The skeletal muscle function of adult zebrafish can also be examined by measuring the contractile strength of intact fish, when electrically stimulated. The determination of the maximum contractile strength is possible through isolated stimuli, while fatigability can be assessed through repeated pulses [73].

Many **microscopic** assays evaluate ALS-related phenotypes, most commonly through the antibody or genetic labelling of motor neurons or NMJ of zebrafish larvae [22, 60, 71, 73, 87]. This allows for the examination of axon length and branching, and colocalization of NMJ terminals. Differences in these endpoints suggest motor axonopathy or NMJ dysfunction, respectively. Immunofluorescence or immunohistochemistry can be used for the labelling of specific ALS-related proteins, such as TDP-43, and evaluation of their subcellular distribution [73, 87]. Many fluorescent probes, such as chloromethyl-2',7'-dihydrodichlorofluorescein diacetate [21], hydroethidine, and mitoSOX [94], can also provide information regarding redox markers and oxidative stress. Additionally, apoptosis in fixed zebrafish embryos or larvae can be assessed using terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) [23] or activated caspase-3 immunofluorescence [96]. In live zebrafish, acridine orange (AO) staining has been used as an alternative method for apoptosis visualisation [97], since it intercalates in the nuclear DNA of apoptotic cells but not living cells [98]. However, AO fluorescence depends on other factors that reduce its selectivity: it tends to accumulate in acidic

compartments such as lysosomes of non-apoptotic cells [99], and its fluorescence spectrum changes with pH [100] and with RNA vs. DNA binding [101].

Other relevant microscopy techniques include transmission electron microscopy, to examine cellular ultrastructure and mitochondrial defects [73], and polarization microscopy, to assess muscular function [22]. The latter takes advantage of the fact that healthy skeletal muscle is highly birefringent due to the structured organization of its myofilaments; birefringence is lost upon muscular degeneration [102].

In conclusion, zebrafish models of ALS offer a wealth of relevant endpoints for the evaluation of their phenotypes. Their thorough characterization is essential for the understanding of each model's similarities and differences to human ALS.

### 2.3. Zebrafish models of ALS

Zebrafish models of ALS have been created using genetic and chemical induction, with varying levels of face and construct validity. This section critically describes established and recently developed zebrafish models of ALS. The nomenclature convention adopted in this section is: lowercase italic zebrafish genes; first letter capitalised, non-italic zebrafish proteins [103]; capitalised italic human genes; capitalised non-italic human proteins [104], as illustrated in Table 1.

**Table 1 – Genetic nomenclature convention for zebrafish and human genes and proteins, illustrated with *FUS* [103, 104].**

| Species   | Gene       | Protein |
|-----------|------------|---------|
| Zebrafish | <i>fus</i> | Fus     |
| Human     | <i>FUS</i> | FUS     |

#### 2.3.1. Genetic models

Approximately half of the familial cases of ALS are associated with mutations in either *C9ORF72*, *SOD1*, *FUS*, or *TARDBP* [38]. As such, most genetically induced zebrafish models of ALS alter the function of these genes, and this section is focused on them. Nevertheless, rare mutations in many other genes have been linked to ALS [105]. Some of them, including *VAPB*, *ELP3* and *ALS2*, have been studied in zebrafish using AMO-mediated knockdown techniques [14]. Fig. 3 represents the characterised pathological phenotypes of the models we present in this section.

*C9ORF72* contains a non-coding hexanucleotide repeat (G4C2) sequence, whose expansion is associated with 22.5% of fALS and 3.1% of sALS cases [38]. The expanded RNA repeats are directly toxic [106], and can also be translated to toxic dipeptide repeat (DPR) proteins [107]. The *C9ORF72* protein is important for the regulation of stress granule dynamics, nucleocytoplasmic import, endosomal trafficking and autophagy [108]. Therefore, its loss of function may also contribute to neurotoxicity [3, 39]. The injection of DNA constructs in the one-cell stage embryo can be used to induce the expression of differently sized G4C2 repeats in zebrafish. Expression of 38× and 72× G4C2 repeats causes increased apoptosis and G4C2 RNA foci in embryo cells, which colocalise with RNA binding proteins [109]. Similarly, RNA microinjection has been used to induce the expression of 3 to 90× sense or antisense G4C2 repeats in zebrafish embryos. Both sense and antisense RNA cause motor axonopathy, including modified,

non-translated, stop-codon-interrupted sequences [110]. These results support the direct toxicity of the expanded RNA repeats, independently of DPR protein toxicity. On the other hand, the stable expression of G4C2-related DPR proteins in zebrafish larvae increases mortality and developmental defects, impairs locomotion, induces DPR protein aggregation and causes motor axonopathy, supporting the role of DPR protein toxicity in *C9ORF72* ALS [78, 111]. Combining RNA toxicity with DPR protein expression, a transgenic line that stably expresses mutant *C9ORF72* containing expanded G4C2 repeats has been developed. These line's larvae show early mortality and swimming impairments, and the adults show muscle atrophy, increased fatigability, heat shock response activation, and motor neuron loss. Both RNA foci and DPR proteins are present in this model [74]. Interestingly, riluzole (an excitotoxicity inhibitor approved for ALS treatment [112]) or ivermectin (an antiparasitic drug that binds to glutamate-gated chlorine channels [113]) reduce the heat shock response in this transgenic model, to similar degrees.

*C9ORF72* loss of function models have also been developed. Loss of function of the zebrafish orthologue of *C9ORF72* through the overexpression of a non-functional mutant or AMO injection leads to axonopathy in the spinal cord and hindbrain, reduces tail coiling at 24 hpf and increases apoptosis in the brain [65]. Similarly, silencing the zebrafish orthologue of *C9ORF72* through the stable expression of specific miRNA leads to increased mortality, impaired synaptic function at the NMJ, motor deficits, muscle atrophy, motor axonopathy and Tdp-43 mislocalization in skeletal muscle [87].

*SOD1* mutations are the second most common in human ALS, having been used to generate animal models of ALS for decades. *SOD1* encodes superoxide dismutase [Cu-Zn] (*SOD1*), a cytoplasmic protein that converts superoxide radicals to less reactive species [114]. *SOD1* mutations are present in 18.9% of fALS and 1.2% of sALS cases; they promote *SOD1* aggregation and the dysregulation of the ubiquitin-proteasome system [3, 38]. Overexpression of three different mutant human *SOD1* alleles in zebrafish, induced through RNA microinjection, results in axonopathy specific to motor neurons [60]. Likewise, transgenic zebrafish larvae that stably express mutant (G93R) *Sod1* have abnormal NMJ, and adults show a progressive loss of endurance, altered muscle response and motor neuron loss. Changes in the number of mitochondria and their integrity were also found in the muscle and motor neurons of G93R *Sod1* mutant larvae [73]. Similar results, together with higher susceptibility to oxidative stress, were found in

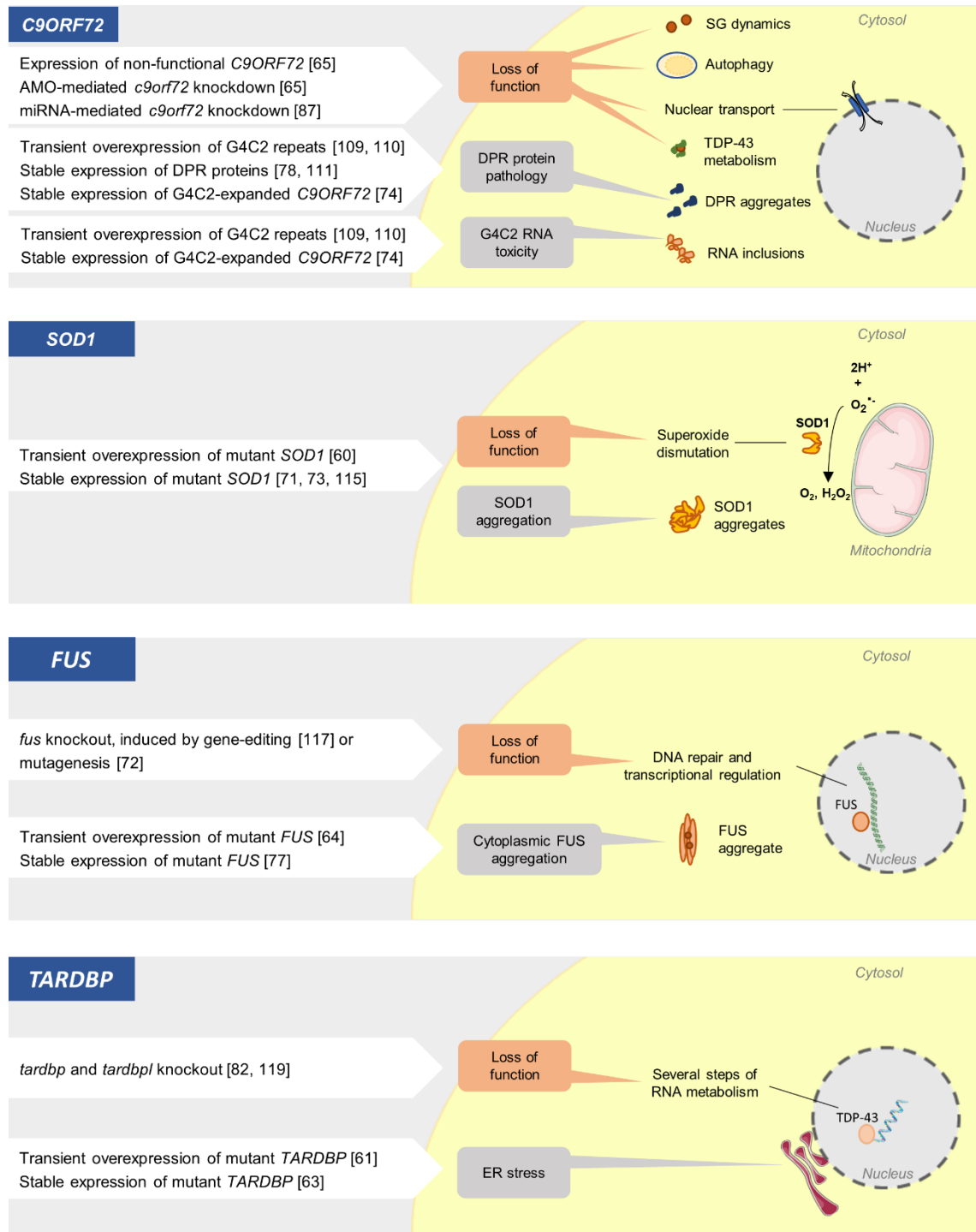


**Fig. 3 – Genetic models of ALS in zebrafish and their associated pathology.** Most genetic models of ALS are based on *C9ORF72*, *SOD1*, *FUS* or *TARDBP*. Each group of models is pointing to their described pathological findings. *C9ORF72* modifications can cause loss of function, affecting stress granule (SG) dynamics, autophagy, nuclear transport and TDP-43 metabolism; dipeptide repeat (DPR) protein pathology; or RNA toxicity. *SOD1* modifications cause loss of function, affecting the dismutation of reactive superoxide produced by the mitochondria; or SOD1 aggregates. *FUS* modifications can cause loss of function, affecting DNA repair and transcriptional regulation; or cytoplasmic FUS aggregation. *TARDBP* modifications can cause loss of function, affecting several steps of RNA metabolism; or increased endoplasmic reticulum (ER) stress.

a T70I Sod1 transgenic zebrafish line [71]. Importantly, mutant *sod1* zebrafish were used to first describe the role of interneuron stress in early ALS [115].

*FUS* mutations are another well described cause of ALS, being present in 3% of fALS and 0.4% of sALS cases [38]. The FUS protein is a nuclear RNA-binding protein that participates in transcriptional regulation and DNA repair [116]. Overexpressed mutant human FUS (R495X or G515X) accumulates in the cytoplasm of zebrafish larvae and, upon heat shock induction, colocalises with stress granules. On the other hand, wild-type human FUS is correctly localised in the nucleus when overexpressed in zebrafish [64]. Stably expressed mutant FUS (R521C) also accumulates in the cytoplasm of cultured zebrafish cells and increases stress granule persistence upon heat shock. In this model, both mutant and wild-type human FUS associate with stress granules [77]. Lastly, although no motor phenotypes were found in AB×TU-strain CRISPR-Cas9-directed *fus* knockout zebrafish [117], a more recent study found impaired touch responses, reduced motor activity, NMJ defects and changes in the alternative splicing of tau, in mutagenesis-generated *fus* knockout AB zebrafish [72]. Thus, the disparity between the CRISPR-Cas9-edited and the mutant *fus* knockouts may be related to their different genetic backgrounds. Overall, loss-of-function mechanisms are likely to be relevant to *FUS*-associated pathology.

The fourth most commonly mutated gene in ALS is *TARDBP*. Mutations in this gene are implicated in 3.3% of fALS and 0.5% of sALS cases [38]. However, the protein it encodes, TDP-43, is the main constituent of the cytosolic protein aggregates found in most patients (FUS- and SOD1-associated ALS cases are the notable exceptions, in which the respective mutant proteins aggregate) [3]. Physiologically, TDP-43 is a DNA- and RNA-binding protein that participates in many steps of RNA metabolism [118]. mRNA microinjection has been used to induce the overexpression of either wild-type or mutant (A315T) human *TARDBP* in zebrafish embryos. *TARDBP* overexpression led to motor axonopathy, which was more pronounced with mutant *TARDBP* than with wild-type *TARDBP* [61]. This motor axonopathy was rescued by co-expression of the neurotrophic factor progranulin. Additionally, impaired reflex responses were found in zebrafish larvae expressing the A315T, G348C, or A382T mutant forms of human TDP-43 [63]. Motor impairments in zebrafish expressing mutant TDP-43 were partially rescued by molecules that reduce endoplasmic reticulum stress (e.g. methylene blue, salubrinal, etc.) [62]. To investigate the role of the loss of normal TDP-43 function in mutant *TARDBP* models, both *TARDBP* ortholog genes (*tardbp* and *tardbpl*) have been knocked out in zebrafish

[82, 119]. Single knockout of *tardbp* is compensated by *tardbpl*, its ohnologue. Double *tardbp* and *tardbpl* knockout zebrafish larvae show phenotypes consistent with ALS, including motor axonopathy, locomotion deficits, muscle degeneration, vascular mispatterning, and early death. Finally, CRISPR-Cas9-based homology-dependent repair has been used to knock-in ALS-relevant point mutations in zebrafish *fus* or *tardbp* genes for the first time [86]. This study showed the feasibility of precisely introducing point mutations in zebrafish models, though further work is needed to characterise the resulting mutants.

In general, genetically induced models of ALS in zebrafish mimic many key features of the human disease – most commonly, motor axonopathy and impaired spontaneous or reflex locomotion. Such models have high construct validity regarding the ALS cases associated with each gene, although only a minority of the patient population bears ALS-causing mutations. Their predictive validity remains unclear: rescue strategies that were effective in zebrafish models have failed in human patients [120], and the effects of approved ALS drugs (riluzole and edaravone) in most zebrafish models have not been characterised. Nevertheless, genetic zebrafish models have contributed to the elucidation of several disease pathways and mechanisms, including the direct toxicity of expanded G4C2 repeat RNA in mutant *C9ORF72* [110], the impact of *FUS* loss-of-function in the NMJ [72] and the role of interneuron stress in early ALS [115].

### 2.3.2. Chemical models

Since most ALS cases are sporadic, where no single mutation is able to explain their aetiology, environmental exposures and toxicogenomic interactions are likely to contribute to this disease, with many different initial mechanisms converging in a common ALS pathway [7]. Thus, chemically induced models may be relevant to human disease even if their construct validity is unclear.

BMAA is the most studied ALS-connected molecule. Given its connection to ALS-PDC clusters [43-45], it has been used as a motor neuron disease inducer in several species [45], including zebrafish. BMAA-exposed zebrafish larvae show concentration-dependent bradycardia, spinal development abnormalities, clonus-like convulsions [19], reduced nerve length, altered NMJ morphology [20], increased oxidative stress [21] and acoustic startle sensitivity [58]. Furthermore, in an interesting model of an ALS-related toxicogenomic interaction, transgenic G93R *sod1* zebrafish exposed to BMAA as embryos show increased fatigability as 5-month-old adults. On the other hand, fish

overexpressing wild-type *sod1* are resistant to phenotypic changes induced by BMAA exposure [20].

Bisphenol A (BPA) exposure has also been proposed as a model for environmentally induced motor neuron degeneration [22]. BPA exposure causes a concentration-dependent increase in mortality, reduced motor responses, reduced motor axon length and branching, reduced NMJ integrity, microglia activation [22], oxidative stress and DNA damage [23]. However, BPA is known to have wide-ranging toxicity [121, 122], which makes BPA-induced models less specific to ALS [123].

Recently, exposure to BMAA was combined with another naturally co-occurring cyanotoxin, microcystin leucine and arginine (MCLR). This combination caused significantly higher increases in acoustic startle sensitivity and Tdp-43 expression than the individual toxins [58]. This is also likely a broader model of the cyanotoxin exposures in humans than simple BMAA exposure.

In conclusion, chemically induced models of ALS may shed light on sporadic forms of this disease, where no single disease-inducing mutation is present. However, especially in the cases of BPA, toxin combinations, and toxicogenomic models, their phenotypes are less well-described than those of most genetic models. Regarding BPA, its current lack of causal connections to human ALS makes its construct validity relatively weak. Thus, the role of BPA exposure as an ALS model hinges on its face and predictive validities, that require a thorough phenotypic characterisation.

## 2.4. Conclusion and future perspectives

The last decade saw a marked increase in interest for zebrafish models of ALS, leading to the current wealth of models and studied phenotypes. However, the phenotypic characterisation of existing models is still incomplete.

Further description of ALS-related phenotypes in zebrafish models is important to solidify their relevance to human disease. ALS patients consistently present protein aggregation in motor neurons, which has yet to be studied in the chemically induced models of sporadic ALS in zebrafish. Additionally, sleep disruptions and cognitive decline in ALS patients are common phenomena which have not been extensively described in zebrafish models of ALS.

Regarding the utility of zebrafish models, higher emphasis should be placed on understanding their predictive validity, which in some cases may surpass that of mice models. For instance, the potential of zebrafish in translational studies is well illustrated by the discovery of the mechanism behind the teratogenicity of thalidomide [124], which does not affect rodent models [125]. Said potential may also be realised in future ALS research.

There are important research opportunities on the generation of new zebrafish models of ALS, in addition to the extended characterisation of existing models. New models may elucidate novel pathophysiological pathways and aetiologies. Recent genome sequencing studies have identified many new genes that are involved in ALS. Those genes can be used to develop novel zebrafish models, which can then become useful tools to understand the mechanistic relevance of those genes [126]. For instance, expression of p53 isoform  $\Delta 133p53$  is decreased in ALS patients [127] – interestingly, loss of function of the zebrafish ortholog of  $\Delta 133p53$  causes accelerated brain aging and senescence through long-term oxidative stress [128]. This may be a potential model for ALS-related oxidative stress if effects on the senescence and degeneration of motor neurons are found. Another possible source of novel ALS models in zebrafish is the expression of endogenous retroviruses. Some of these loci are upregulated in tissues of ALS patients [129], and the expression of human endogenous retrovirus K (HERV-K) has been found to cause neuron retraction *in vitro* and ALS-consistent motor deficiencies in model mice. Additionally, HERV-K expression is regulated by TDP-43, the main constituent of protein aggregates in most ALS patients [46]. If equivalent results are found upon HERV-K overexpression in zebrafish, it has the potential to be another useful model for

fundamental research and drug discovery and development. Furthermore, since toxicogenomic interactions are highly relevant to the aetiology of ALS [7], the generation of models by exposing transgenic or mutant animals to chemical inductors of neurodegeneration can be an interesting tool. Toxin combinations may also be used to reflect natural toxic exposures in ALS patients [58], possibly allowing for the generation of highly relevant zebrafish models.

In conclusion, zebrafish models of ALS have many advantages in tractability and assay throughput, making them interesting tools for fundamental and, potentially, translational research. There is a wide array of ALS-relevant phenotypes in zebrafish, although they are not standardised and had not been previously aggregated in the literature. There are plentiful research opportunities in the study of existing ALS models – in the expansion of their characterisation, understanding of the underlying pathological mechanisms, and testing of novel rescue strategies – and in the development of new models, towards a deeper understanding of ALS, its pathophysiology and treatment avenues.





### 3. Original Research

#### **Behavioural and Molecular Analysis of Chemically Induced Zebrafish Models of Amyotrophic Lateral Sclerosis**

##### **Abstract**

Amyotrophic lateral sclerosis (ALS) is a progressive and devastating neurodegenerative disease, in which the death of motor neurons leads to loss of muscle function. Additionally, cognitive decline and sleep disruptions are common in ALS patients, contributing to disease progression and burden. Most ALS cases are idiopathic, and environmental exposures likely contribute to sporadic disease. The small vertebrate zebrafish is a leading organism to model neurodegenerative diseases; past behavioural characterization of zebrafish models has been primarily focused on spontaneous locomotion and reflexes. Previous studies have proposed bisphenol A (BPA) and  $\beta$ -methylamino-L-alanine (BMAA) exposure to model sporadic ALS in zebrafish, inducing motor neuron damage and altered motor responses. Here we innovate by extending the phenotypic characterisation of the BPA and BMAA models, including motor activity, sleep, redox, and proteostasis phenotypes, to assess their face validity for ALS drug development. We also assess their predictive validity by combining BPA or BMAA exposures with approved ALS drugs. We used an infrared-based Locomotion Activity Monitor (LAM) to characterise zebrafish motor activity and sleep in BPA or BMAA-exposed animals versus non-exposed controls. We also evaluated the effect of BPA and BMAA in embryo tail coiling activity and in motor neuron morphology, and further describe the impact of BPA on oxidative damage and levels of proteins involved in ALS pathology. BPA exposure induced concentration-dependent reductions in motor activity and increased sleep ratio, while BMAA caused a reduction in tail coiling activity; BPA also increased oxidative damage and altered the levels of mitochondrial proteins. Additionally, the approved ALS drug edaravone, but not riluzole, partially rescued BPA-induced motor phenotypes. Overall, BPA exposure is a promising model of ALS-related oxidative damage through mitochondrial dysfunction. These results evidence the feasibility of combining the LAM with molecular studies, to assess multi-parameter disruptions in zebrafish models of neurodegeneration. Furthermore, the extended characterization of sporadic ALS models, such as those induced by BPA or BMAA, can assist future studies addressing pathogenic pathways and experimental therapeutics.

### 3.1. Introduction

Amyotrophic Lateral Sclerosis (ALS) is a progressive and fatal neurodegenerative condition. In ALS patients, the degeneration of upper and lower motor neurons leads to loss of motor function, muscle wasting, and death [6]. Additionally, frontotemporal neurons and other neuron populations are also frequently affected, causing cognitive, behavioural, or sleep impairments in approximately half of the patients [29, 33]. ALS is the most common motor neuron disease [14], imposing a high burden to patients, society and healthcare systems [5]. The pathophysiology of ALS is highly complex, including loss of protein and RNA homeostasis [37], mitochondrial dysfunction, excitotoxicity, oxidative stress, neuroinflammation, glial dysfunction, and several other pathological mechanisms [3, 39, 50]. These mechanisms lead to the loss of axonal projections, neuronal death and muscle denervation that characterise ALS [3]. While most ALS cases are sporadic, 5–20% have hereditary (familial) causes. Nevertheless, ALS is highly heterogeneous, and its aetiology likely involves both genetic and environmental factors [3, 7].

There is a need for new and more effective treatments for ALS, given that currently FDA-approved drugs riluzole (an excitotoxicity inhibitor [112]) and edaravone (a free radical scavenger [112]) provide only modest improvements to patients [26–28]. Development of new therapies, in turn, requires appropriate model systems, namely *in vitro* and *in vivo* models of ALS [53]. In that regard, zebrafish (*Danio rerio*) is a promising model animal, given its unique combination of experimental tractability with biological relevance [10, 11, 14, 130].

Several zebrafish models of ALS have been described in the last decades, mostly based on genetic manipulation of one of the causative genes of familial ALS (fALS) [61, 64, 65, 71–74, 87, 109, 110]. Additionally, to model the more common sporadic ALS (sALS), exposure to the ubiquitous contaminant bisphenol A (BPA) [22, 23] or to cyanotoxins linked to clusters of motor neuron disease [43–45] (such as  $\beta$ -methylamino-L-alanine; BMAA) [20, 21, 58] have been proposed. Concretely, neurotoxicity mechanisms of BPA that are relevant to the pathophysiology of ALS include oxidative stress [23, 131–134] and mitochondrial disruption [135]; while BMAA is known to induce excitotoxicity, possibly disturb proteostasis [49], and inhibit the synthesis of the neuroprotector and sleep-regulating hormone melatonin [136].

Here, we study the previously proposed BPA- and BMAA-induced zebrafish models of sALS, with the aim of furthering the description of their face and predictive validity for the development of ALS therapies. We extend the characterisation of motor, sleep, and pathophysiological phenotypes, and propose mechanisms for BPA neurotoxicity. Furthermore, we assess the effectiveness of approved ALS drugs in rescuing motor phenotypes.

### 3.2. Methods

#### 3.2.1. Drugs and treatment solutions

Stock solutions of 44 mM BPA (Sigma-Aldrich), 50 mM edaravone (Abcam) and 10 mM riluzole (MedChemExpress) were prepared in dimethyl sulphoxide (DMSO, Sigma-Aldrich); the stock solution of 60 mM BMAA (Tocris) was prepared in distilled water. Treatment solutions for zebrafish experiments were prepared by diluting the stocks in dechlorinated water. In each experiment, all conditions had the same DMSO concentration, which was kept below 0.5% (1% for the riluzole and edaravone survival studies).

#### 3.2.2. Zebrafish maintenance, husbandry, and drug incubation

Adult AB-strain zebrafish (*Danio rerio*), supplied by the Interdisciplinary Centre of Marine and Environmental Research (CIIMAR, Portugal), were maintained at  $28 \pm 1$  °C, under 14 h–10 h light-dark cycles. Eggs were produced from these zebrafish as previously described [88]. Briefly, on the afternoon before egg collection, individual couples were placed in 0.7 L breeding tanks, where water from the main tank circulated. On the following morning, 1 hour after the start of the light phase, the eggs were collected, washed and maintained in E3 medium (5.0 mM NaCl, 0.17 mM KCl, 0.33 mM  $\text{CaCl}_2 \cdot 2 \text{H}_2\text{O}$ , 0.40 mM  $\text{MgCl}_2 \cdot 6 \text{H}_2\text{O}$ , 0.01% Methylene blue [137]) at 28 °C. At 4 hours post-fertilization (hpf), eggs were exposed to drugs in 24-well plates (5 embryos/500  $\mu\text{L}$ /well, for survival or behavioural studies) or 12-well plates (15 embryos/1000  $\mu\text{L}$ /well, for immunofluorescence or protein extraction). Dead embryos were removed daily. In locomotor activity monitoring experiments, half volume was replaced with freshly prepared treatment solutions 24 hours before monitoring began.

#### 3.2.3. Behavioural assays

##### Tail coiling activity assay

For tail coiling studies,  $25 \pm 1$  hpf embryos were individually transferred to a custom-made laser-cut 36-well plate (2 mm well diameter) (Fig.1A), filled with the respective treatment solutions. After a 5-minute acclimatization period, each embryo was recorded for 3 minutes (4 frames per second), with a 4 $\times$  magnification objective, using the previously described custom Nikon-Prior-Hamamatsu (NPH) Imaging System [89, 138] in a dark-field configuration.

The videos were analysed with the Fiji distribution of ImageJ2 [139] using a custom macro, based on a previously described analysis pipeline [140] (Fig. 4A). Briefly, the video frames were converted to 8-bit images, and then subjected to Fiji's Gaussian blur, Smooth and Find Edges. The video stack was duplicated, then shifted by one frame and subtracted to the duplicate, to generate a stack of the differences between each frame. Next, the Z-axis profile of the mean intensity of the resulting stack was plotted, and the BAR plugin [141] was used to find intensity peaks, corresponding to tail coiling movements. The data were exported to a .csv file and the peaks were automatically counted using Microsoft 365 Excel.

To validate the automated counting method, we randomly selected 30 videos from 3 different experiments, counted them first manually and then using the Fiji macro, and compared the results (Fig. 4B).

### Locomotor activity monitoring

For the activity studies with zebrafish larvae, the infrared-based Locomotor Activity Monitor (LAM model LAM10, Trikinetics Inc.) setup was used as previously described [88]. Briefly, 4-days-post-fertilisation (for the experiments with BPA alone) or 5-days-post-fertilisation (for the BPA/edaravone combination or BMAA experiments) larvae

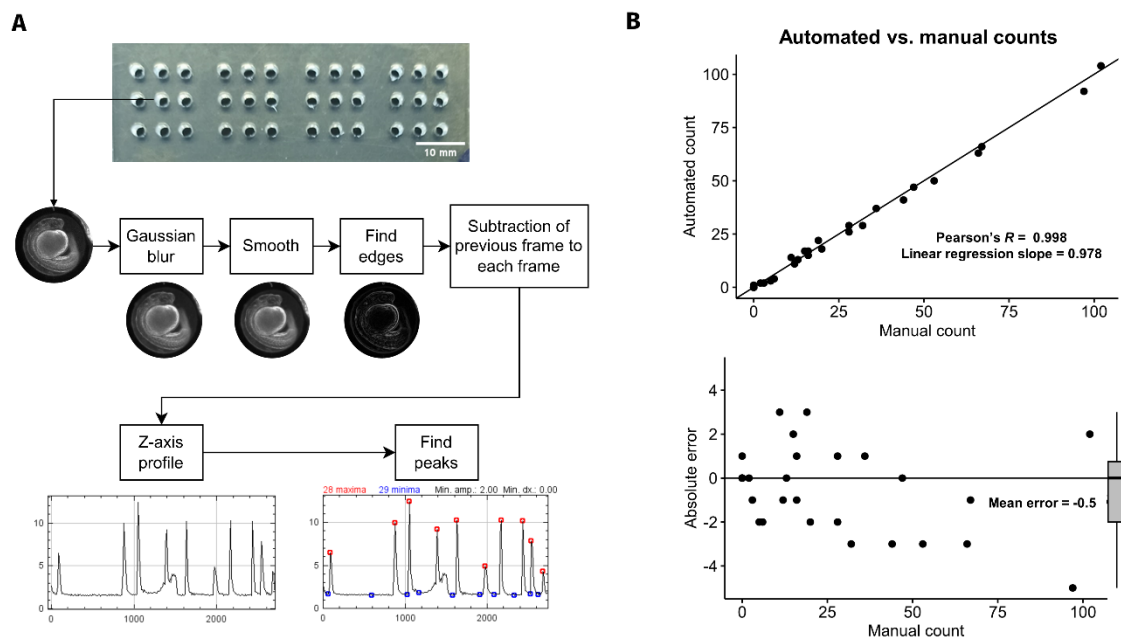


**Fig. 4 – Algorithm for automated tail coiling counting.** (A) Workflow of the Fiji macro used for counting tail coiling movements. A time series stack of the zebrafish embryo is acquired using the custom 36-well plate, then processed with *Gaussian blur*, *Smooth*, and *Find edges*. The processed stack is duplicated, shifted by one frame, and subtracted to the original stack. A Z-axis profile is then generated from the result, and peaks are counted using BAR's *Find peaks*. (B) Validation of the method for tail coiling counting: (top) results obtained by the automated counting method vs. results from manual counts; (bottom) distribution of the automated counting errors.

were transferred to LAM tubes filled with either dechlorinated water or treatment solution, with a 100  $\mu$ L air bubble. The tubes were placed in the LAM, and the setup was maintained at 28 °C, under a 14h–10h light-dark cycle. Larvae activity was recorded every 30 seconds as the count of infrared beam crossings in each tube, for up to 96 hours. LAM data were analysed with the free and open source Rtivity software [142], developed by our group, which computed the parameters related with activity and sleep, as previously detailed [88]. The inactivity threshold for sleep quantification was set at 10 minutes, while the threshold for activity bout quantification was set at 2 minutes.

### 3.2.4. Birefringence imaging

For birefringence imaging, 8 dpf zebrafish larvae were anaesthetised with 0.02% tricaine and imaged as previously described [102]. Briefly, two orthogonally oriented linear polarising filters (ScreenTech) were used to adapt the aforementioned NPH Imaging System [89] for polarised light microscopy, with a 4 $\times$  magnification objective. Zebrafish larvae orientation, relative to the plane of polarisation, was optimised for maximum birefringence and kept identical for all larvae.

### 3.2.5. Immunofluorescence

3 dpf zebrafish larvae were processed for immunofluorescence as we previously described [143]. Briefly, after fixation with paraformaldehyde, they were depigmented and permeabilized with a solution of H<sub>2</sub>O<sub>2</sub> and KOH. Next, the larvae were blocked, incubated with the anti-acetylated- $\alpha$ -tubulin primary antibody (T6793, Sigma-Aldrich; 1:1000, 24 h), washed, and incubated with the respective Alexa-488-conjugated secondary antibody (A-11034, Life Technologies; 1:500, 24 h). After washing the secondary antibody, the larvae were incubated for 30 minutes with 1  $\mu$ g/mL Hoechst 34580, a nuclear stain. They were then washed with PBS (3 times, 5 minutes) and mounted with Glycergel medium (DAKO). Imaging was conducted with a 10 $\times$  magnification objective, using the same custom NPH Imaging System [89], with a DAPI/FITC/TRITC/CY5 Quad band-pass filter (Chroma). Excitation wavelengths were 488 nm for alexa-488 and 380 nm for Hoechst.

### Analysis of motor axon development

Motor axons of larvae immunostained for acetylated  $\alpha$ -tubulin were examined for morphological abnormalities. For each larva, 12–22 ventral motor axons from the posterior half of the body were scored for defects, based on previously described classification methods [144, 145] (Table 2); the average proportion of axons with each score was compared between control and treated fish. The most caudal axon on each side was not considered, given its highly variable morphology. Axonal length was also evaluated using Fiji, as previously described [60], for the caudal-most 6<sup>th</sup> to 10<sup>th</sup> ventral motor axons of each larva. Axon lengths were measured from the point of separation between the ventral and dorsal axons to the distal tip of the ventral axon. All immunofluorescence image analyses were conducted with blinding to treatment conditions.

**Table 2 – Classification of ventral motor neuron morphology.** Adapted from previously described classification schemes [144, 145].

| Classification   | Axon morphology                                                                     |
|------------------|-------------------------------------------------------------------------------------|
| Severe defects   | Complete absence or early truncation of the axon                                    |
| Moderate defects | Excessive branching or innervation of the neighbouring muscle segment               |
| Mild defects     | Defasciculated axon or lack of stereotypical morphology without more severe defects |
| No defects       | Normal                                                                              |

### 3.2.6. Western and dot blots

#### Protein extraction and quantification

Protein extraction and quantification was performed as we previously described [146]. 30 larvae per condition were washed and transferred to ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 1 mM EDTA) supplemented with protease inhibitor cocktail (Thermo Scientific). After 3 freeze-thaw cycles and homogenisation (Precellys Evolution, 6800 rpm, 5 × 42 s with 37 s pauses, CK28 beads), lysates were centrifuged at 12,000 × g (10 min, 4 °C) and supernatant protein concentrations were quantified by the Bradford method.

**Western blot**

Western blotting was conducted as we previously described [146]. After total protein quantification, samples were denatured (95 °C, 5 min) in NuPAGE LDS sample buffer (Invitrogen) supplemented with Bolt sample reducing agent (Novex), loaded (30 µg protein) into 8-16% Mini-PROTEAN TGX pre-cast SDS-polyacrylamide gels (BioRad), electrophoresed (125 V, 75 min), and transferred into PVDF membranes (overnight, 30 V, 4 °C). Membranes were then blocked (1 h, 4 °C) with 5% bovine serum albumin (BSA, NZYTech) in PBS with 0.05% Tween 20 (PBST), and incubated with primary antibodies, according to Table 3. After washing with PBST, membranes were incubated for 1 h with appropriate secondary antibodies conjugated with horseradish peroxidase (G-21234, 1:4000, Thermo Scientific; G-21040, 1:4000, Invitrogen), for detection using Novex ECL Chemiluminescent kit (Invitrogen) and a ChemiDoc MP Imaging System (Bio-Rad). Membrane Coomassie (Coomassie Brilliant Blue G-250, Thermo Scientific) staining was used as loading control. Densitometric analyses were performed with Fiji.

**Table 3 – Primary antibodies used for immunoblots.**

| <b>Target</b> | <b>Antibody</b> | <b>Dilution</b> | <b>Incubation time</b> | <b>Source</b> |
|---------------|-----------------|-----------------|------------------------|---------------|
| Hsp70         | sc-66049        | 1:1000          | 1 hour                 | Santa Cruz    |
| Hsp60         | 15282-1-AP      | 1:4000          | 1 hour                 | Proteintech   |
| Grp75         | 14887-1-AP      | 1:1000          | 1 hour                 | Proteintech   |
| Sdha          | ab66484         | 1:2000          | 1 hour                 | Abcam         |
| Grp78/BiP     | 11587-1-AP      | 1:6000          | 1 hour                 | Proteintech   |
| Ubiquitin     | sc-8017         | 1:500           | 1 hour                 | Santa Cruz    |
| 4-HNE         | ab46545         | 1:1000          | Overnight              | Abcam         |

**Quantification of 4-HNE protein adducts**

After total protein quantification, samples containing 25 µg of protein were spotted on a nitrocellulose membrane (Bio-Dot Microfiltration Apparatus, Bio-Rad), as we previously described [89]. Membranes were blocked for 1 h with 5% BSA in PBST and incubated with anti-4-HNE antibody (ab46545, 1:1000, Sigma-Aldrich; overnight). After washing with PBST, membranes were incubated with secondary antibody (G-21234,

1:4000, Thermo Scientific, 1 h), and imaged as described for western blots. Coomassie staining was used for loading control. Densitometric analyses were performed with Fiji.

### **3.2.7. Data analysis**

Data are shown as mean  $\pm$  standard error of the mean (SEM) of the  $n$  number indicated in figure legends, except for zebrafish survival data that are shown as Kaplan-Meier representations. Data from zebrafish larvae are from at least 3 different breedings; exceptions are noted in the figure legends and interpreted as preliminary data. When applicable, concentration-response curves were fitted using either a quadratic or a 4-parameter log-logistic model (the best model was selected using Akaike's information criterion). Normality assumptions were tested with the Shapiro-Wilk test; for normally distributed data, we used Student's one-sample  $t$ -test when testing whether the mean of one group is different from zero, Student's two-sample  $t$ -test when comparing the means of two groups, ANOVA with Dunnett's post-hoc when comparing two or more groups to a control group, and ANOVA with Tukey's post-hoc when comparing three or more groups to each other. For non-normally distributed data, we used the Kruskal-Wallis test with Dunn's post-hoc. To compare survival curves, we used the log-rank test followed by Cox's proportional hazards regression.  $p$  values under .05 were considered statistically significant. Data analyses were performed with R 4.1.2 [147] (Rstudio 2021.09.1), using the packages listed in Table 4.

**Table 4 – R packages used for data analysis.**

| <b>Package</b> | <b>Use</b>                                                         | <b>Version</b> | <b>Reference</b> |
|----------------|--------------------------------------------------------------------|----------------|------------------|
| readxl         | Importing Excel files                                              | 1.3.1          | [148]            |
| tidyverse      | Data import, tidying, manipulation, visualisation, and programming | 1.3.1          | [149]            |
| rstatix        | Basic statistical tests                                            | 0.7.0          | [150]            |
| DescTools      | Additional statistical tests and descriptive statistics tools      | 0.99.44        | [151]            |
| survival       | Survival analysis                                                  | 3.2-13         | [152, 153]       |
| survminer      | Drawing survival curves                                            | 0.4.9          | [154]            |
| drc            | Dose-response analysis and visualisation                           | 3.0-1          | [155]            |
| cowplot        | Plot annotations and arranging plots into a grid                   | 1.1.1          | [156]            |
| ggpubr         | Data visualisation in publication-ready graphs                     | 0.4.0          | [157]            |
| ggetho         | Visualisation of high-throughput behavioural data                  | 0.3.6          | [158]            |
| ggExtra        | Drawing marginal plots on primary plots                            | 0.10.0         | [159]            |

### 3.3. Results

#### 3.3.1. Effects of BPA exposure on zebrafish behaviour and muscle organisation

Loss of motor function is a hallmark of ALS, caused by the degeneration of motor neurons [3]. To assess whether sublethal BPA exposure causes behavioural changes consistent with ALS in zebrafish, we quantified its effects on survival, spontaneous locomotor activity and sleep. The highest concentration of BPA that did not increase mortality was 20  $\mu$ M (Fig. 5A), so it was the highest concentration used for locomotor activity analysis. Larvae (4–6 dpf) continuously treated with 10 or 20  $\mu$ M BPA showed significantly higher sleep ratio and lower activity, activity bout duration, and activity per bout, compared with controls. In general, the effects of BPA exposure were greater during the light phases than during the dark phases of the day (Fig. 5B). Control and BPA-treated larvae showed globally similar activity-over-time profiles, with higher activity during the light phases; however, in 6 dpf larvae, BPA treatment delayed peak activity (Fig. 5B-v).

Additionally, we measured the impact of BPA exposure on the frequency of tail coiling movements in 1-day-old embryos. Tail coiling movements are spontaneous contractions of the embryo tail, mediated by a spinal neural circuit [160], which are altered by neurotoxic exposures [161]. Embryos exposed to 60  $\mu$ M BPA, but not lower concentrations, showed a significant reduction in tail coiling activity (Fig. 5C).

Finally, to assess the presence of gross muscle defects, that could cause locomotor hypoactivity in zebrafish larvae, we verified that 20  $\mu$ M BPA did not affect muscle birefringence, indicative of skeletal muscle organisation (Fig. 5D).

#### 3.3.2. Effects of BPA exposure on motor neuron morphology

Motor neurons are the main affected neuron population in ALS [3]. To understand whether BPA-exposed zebrafish show changes in motor neuron length and morphology (motor axonopathy), we used immunofluorescence to observe the motor neurons of treated and control 3 dpf larvae. The incidence of morphological abnormalities was similar in BPA-treated and control larvae. Preliminary data indicate that BPA exposure may reduce motor axon length, though statistical significance was not reached (Fig. 6A).

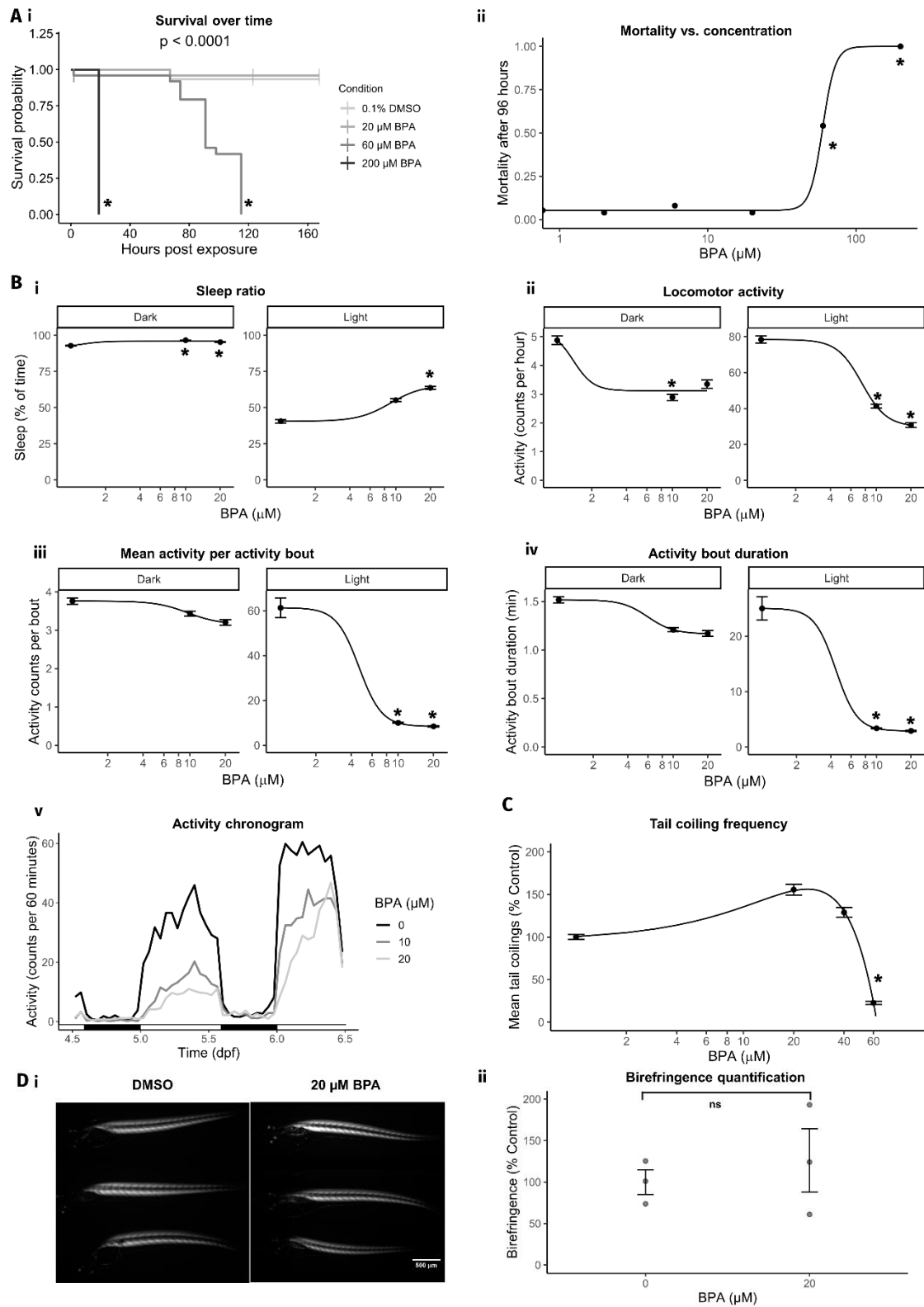


**Fig. 5 – Effects of BPA exposure on survival, behaviour and muscle organisation.** (A) Effect of BPA on zebrafish survival: (i) Kaplan-Meier survival representation of zebrafish larvae exposed to selected BPA concentrations,  $p$  value on graph from the global log-rank test; (ii) mortality after 96 hours of exposure, per BPA concentration;  $n = 25\text{--}30$  larvae; \*  $p < .05$  vs. solvent control, Cox's proportional hazards regression. (B) Results from the infrared-based locomotor activity monitor in zebrafish larvae, from 4 to 6 dpf, in the light and dark phases of the day-night cycle: (i) sleep ratio, i.e. proportion of time that was spent sleeping; (ii) mean locomotor activity counts per hour; (iii) mean number of activity counts per activity bout; (iv) mean duration of activity bouts; (v) mean activity over time; black and white annotation on the x-axis corresponds to dark and light phases of the day, respectively;  $n = 21$  larvae. \*  $p < .05$  vs. control, Kruskal-Wallis test with Dunn's post-hoc. (C) Tail coiling frequency of 1 dpf zebrafish embryos;  $n = 21\text{--}27$  embryos. Data in percentage of the mean of the respective solvent control. \*  $p < .05$  vs. control, Kruskal-Wallis test with Dunn's post-hoc. (D) Effect of BPA on the muscle birefringence of 8 dpf larvae: (i) birefringence imaging of BPA-treated vs. control larvae show no differences in gross muscle organization; scale bar, 500  $\mu\text{m}$ ; (ii) birefringence intensity quantification;  $n = 3$  larvae, all from the same breeding. Data in percentage of the mean of the respective solvent control. Two-sample Student's  $t$ -test.

### 3.3.3. Oxidative damage, proteostasis and mitochondrial proteins

To characterise the mechanisms mediating BPA-induced hypoactivity, we compared markers of oxidative damage, proteostasis, autophagy, and levels of key mitochondrial proteins in zebrafish exposed to BPA, versus control animals. Protein extracts from zebrafish larvae treated with 20  $\mu\text{M}$  BPA showed higher levels of the lipid peroxidation marker 4-hydroxynonenal (4-HNE) (Fig. 6B). Additionally, in larvae treated with 10  $\mu\text{M}$  BPA, we observed a significant increase in the absolute levels of the mitochondrial chaperone Hsp60, but not in the ratio of Hsp60 to Sdha (a constitutively expressed subunit of the mitochondrial respiratory chain complex II). Data indicated no significant differences regarding the levels of Hsp70, a master regulator of protein degradation [162]; Grp75, the mitochondrial isoform of Hsp70 [33]; Grp78, the endoplasmic reticulum (ER) isoform of Hsp70 [163]; protein-bound ubiquitin, involved in protein degradation [162]; free and lipidated LC3 (LC3-I and LC3-II, respectively), involved in autophagy [164]; and Sdha (Fig. 6B).

### 3.3.4. Rescue of BPA-induced hypoactivity with ALS drugs

Edaravone and riluzole are the two FDA-approved drugs for ALS treatment [112]. To assess the predictive validity of the BPA-induced model of ALS, we tested the rescue of BPA-induced hypoactivity with those two drugs. Riluzole and edaravone concentrations were chosen according to previous studies [165, 166], and the bioactivity of the drugs was confirmed through survival and morphological studies (Supplemental Fig. 1). We quantified spontaneous motor activity in zebrafish treated with BPA and edaravone, alone or in combination, regarding tail coiling at 1 dpf and locomotor activity at 5 dpf. Exposure to 60  $\mu\text{M}$  BPA or 100  $\mu\text{M}$  edaravone did not significantly decrease tail coiling activity.

However, larvae that had been exposed to the combination of edaravone and BPA showed significantly higher tail coiling activity than larvae exposed only to BPA (Fig. 7A). On the other hand, 20  $\mu$ M BPA, 100  $\mu$ M edaravone, and their combination did not significantly alter locomotor activity at 5 dpf, compared to the control (Fig. 7B). We also evaluated the effect of 0.5  $\mu$ M riluzole in combination with BPA on tail coiling frequency and found no significant differences in the preliminary data (Fig. 7C).

## 3.3.5. Behavioural and motor neuron effects of BMAA exposure in zebrafish

Since BMAA exposure has been connected to ALS in human populations and animal models [49], we also evaluated its effect on zebrafish activity. No excess mortality was induced by 0.1–3.0 mM BMAA exposure (Fig. 8A). Zebrafish exposed to 1 mM BMAA

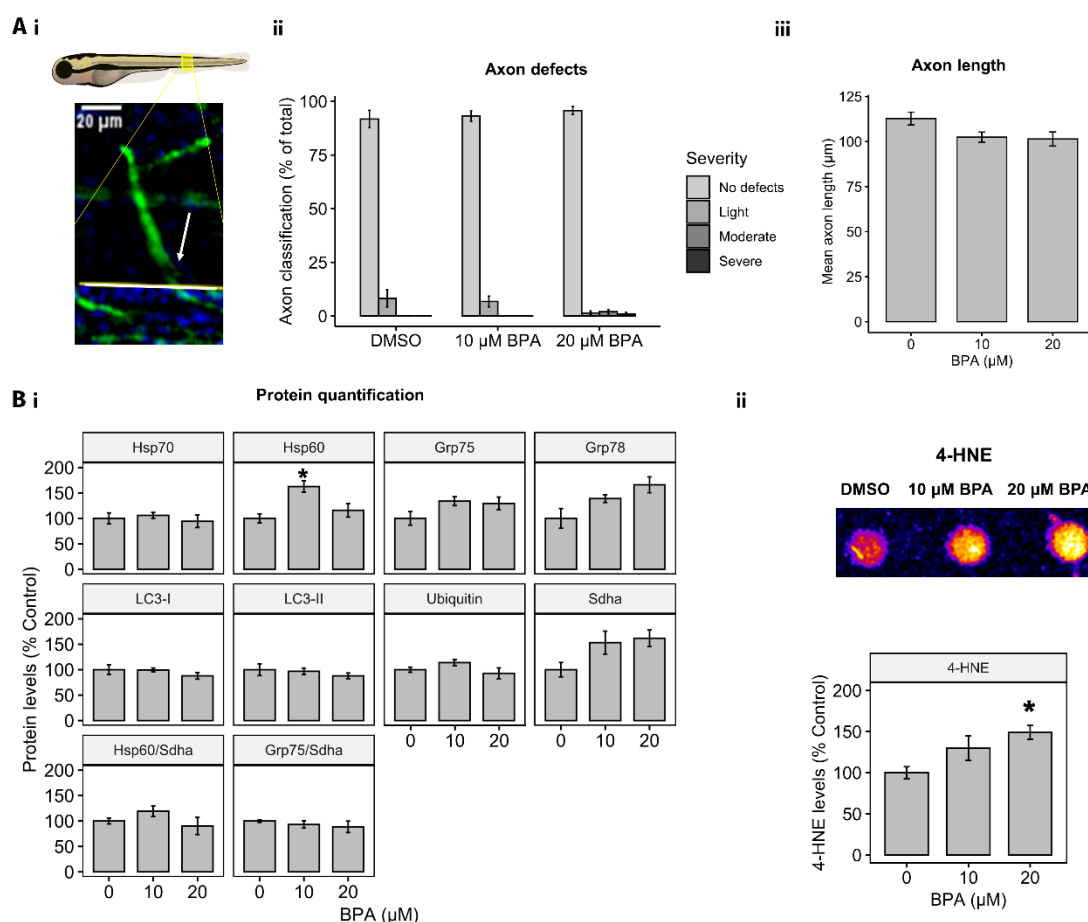


**Fig. 6 – Effects of BPA exposure on motor axons and protein levels.** (A) Assessment of motor neuron morphology and length in 3 dpf zebrafish larvae: (i) representative image of a defective motor axon; scale bar, 20  $\mu$ m. Green: acetylated  $\alpha$ -tubulin. Blue: nuclei. White line: ventral edge of the notochord. Arrow: a ventral motor axon that branches prematurely, above the white line. Original zebrafish larva drawing by Lizzy Griffiths [1]; all other images and data are from the authors' laboratory; (ii) proportion of motor axons with each classification, per condition, as a percentage of the evaluated axons; (iii) mean axon length of each fish, per BPA concentration;  $n = 7$ –10 larvae, all from the same breeding. (B) Quantification of proteins and 4-HNE in 5 dpf whole-larva zebrafish protein extract: (i) results of densitometric analysis for the evaluated proteins, per condition;  $n = 3$  independent replicates; (ii) representative dot blot and results of densitometric analysis for 4-HNE;  $n = 4$  independent replicates. Data in percentage of the mean of the solvent control. \*  $p < .05$  vs. control, ANOVA with Dunnett's post-hoc.

showed significantly lower tail coiling activity at 1 dpf (Fig. 8B), but no significant difference in daytime sleep ratio, activity, activity bout duration or activity per bout at 5 dpf (Fig. 8C).

As with BPA-exposed zebrafish, we used immunofluorescence to observe the motor neurons of BMAA-treated and control 3 dpf larvae. Larvae exposed to BMAA did not show a significantly higher incidence of morphological abnormalities or significantly shorter motor axons than control larvae; rather, exposure to 1000  $\mu$ M BMAA caused a statistically significant increase in motor neuron length (Fig. 8D).

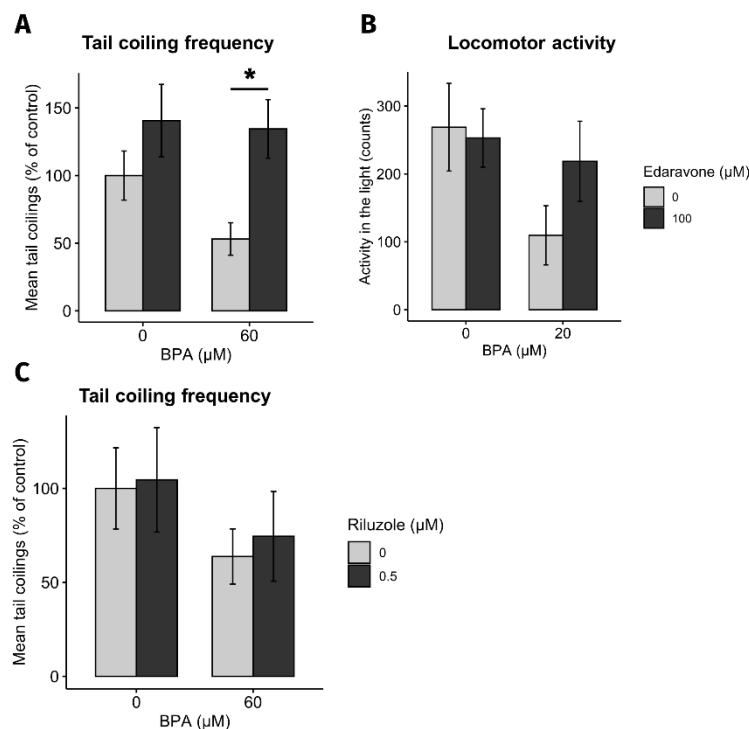


**Fig. 7 – Combination of BPA with FDA-approved ALS drugs.** (A) Combination of edaravone with BPA, tail coiling frequency of 1 dpf zebrafish embryos,  $n = 24$ –27 embryos. Data in percentage of the mean of the respective solvent control. (B) Combination of edaravone with BPA, results of the infrared-based locomotor activity monitor in 5 dpf zebrafish larvae, in the light phase of the day-night cycle,  $n = 16$  larvae from 2 breedings. (C) Combination of riluzole with BPA, tail coiling frequency of 1 dpf embryos,  $n = 16$ –18 embryos from 2 breedings. Data in percentage of the mean of the respective solvent control. \*  $p < .05$ , Kruskal-Wallis test with Dunn's post-hoc.

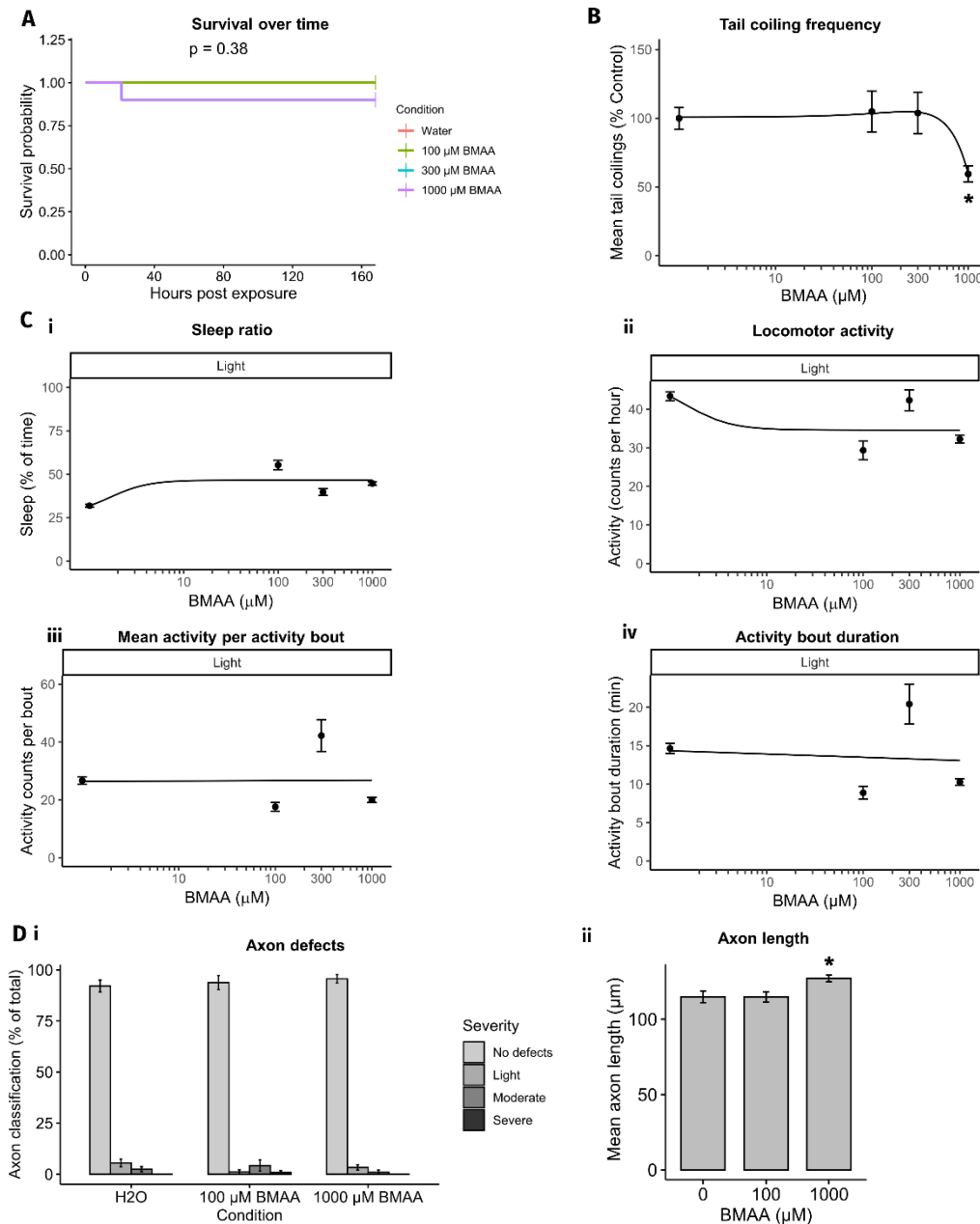


**Fig. 8 – Effects of BMAA exposure on survival, behaviour, and motor neurons.** (A) Kaplan-Meier survival representation of zebrafish larvae exposed to BMAA;  $n = 10\text{--}20$  larvae;  $p$  value on graph from the global log-rank test. (B) Tail coiling frequency of 1 dpf zebrafish embryos;  $n = 18\text{--}26$  embryos. Data in percentage of the mean of the respective solvent control. \*  $p < .05$  vs. control, Kruskal-Wallis test with Dunn's post-hoc. (C) Results from the infrared-based locomotor activity monitor in 5 dpf zebrafish larvae, in the light phase of the day-night cycle: (i) sleep ratio, i.e. proportion of time that was spent sleeping; (ii) mean locomotor activity counts per hour; (iii) mean number of activity counts per activity bout; (iv) mean duration of activity bouts;  $n = 18\text{--}25$  larvae; Kruskal-Wallis with Dunn's post-hoc. (D) Assessment of motor neuron morphology and length in 3 dpf zebrafish larvae: (i) proportion of motor axons with each classification, per condition, as a percentage of the evaluated axons; (ii) mean axon length of each fish, per BMAA concentration;  $n = 7\text{--}10$  larvae, all from the same breeding. \*  $p < .05$  vs. control, ANOVA with Dunnett's post-hoc.

### 3.4. Discussion

The validation of biologically relevant and experimentally tractable sALS models is crucial for the development of new therapies. Here, we aimed to extend the characterisation of ALS-consistent motor and biochemical phenotypes in chemically induced zebrafish models. We found relevant changes in motor activity, oxidative damage markers and protein levels, which contribute to these models' face validity. We also described the effect of approved ALS drugs on motor phenotypes in these models, adding to the understanding of their predictive validity.

#### 3.4.1. Activity and sleep phenotypes

Motor dysfunction is the main clinical manifestation of ALS, presenting as spasticity, weakness, twitching, cramping, and muscle wasting [3]. Death usually results from respiratory failure, due to dysfunction of the muscles involved in breathing [167]. Thus, the revised ALS functional rating score (ALSFRS-R) [168], an often-used endpoint in clinical trials of ALS therapies [169, 170], includes an evaluation of performance in several motor tasks. Since the efficacy of ALS treatment hinges on improving motor function, robust and well characterised motor phenotypes are highly relevant for preclinical models used in ALS drug development [120]. Here, we show such phenotypes in two chemically induced zebrafish models of ALS.

Our data show a reduction in tail coiling frequency in zebrafish embryos exposed to either BPA or BMAA. Tail coiling movements are mediated by spinal motor neurons [160], commonly affected in ALS, and decreases in their frequency have been linked to impaired neurogenesis [161]. Therefore, the decreased tail coiling activity that we observed is consistent with an ALS phenotype. However, in the case of the BPA-induced model, the concentration that affects tail coiling activity (60  $\mu$ M) significantly increases mortality over the following days. While 15  $\mu$ M BPA has been previously reported to decrease tail coiling frequency at 27–29 hpf [116], we did not see tail coiling frequency alterations at concentrations of BPA below 60  $\mu$ M, at the studied time point ( $25 \pm 1$  hpf). Since we showed that 60  $\mu$ M BPA increases mortality after 96 hours, unspecific developmental toxicity (possibly due to the estrogenic effects of BPA [171]) might be implicated in the tail coiling phenotype induced by this higher concentration. Furthermore, lower (15–20  $\mu$ M) BPA concentrations may exert their neurotoxicity after longer exposure times, consistently with the previous report of decreased tail coiling

frequency (at 27–29 hpf) [116] and our LAM results (at 4–6 dpf). From this perspective, the reduction in tail coiling frequency that we observed in embryos exposed to 1 mM BMAA is likely to be more specific and relevant for ALS modelling than that of 60  $\mu$ M BPA, since that concentration of BMAA did not induce excess mortality. That is consistent with studies showing unspecific cell death in BPA-treated (50  $\mu$ M) zebrafish [22], and specific neurotoxicity of BMAA [20, 45]. Our results suggest that tail coiling assays on BPA or BMAA-exposed zebrafish embryos may be useful preclinical endpoints for sALS studies; however, the precise mechanisms implicated on tail coiling phenotypes still require further elucidation.

Several hypoactivity phenotypes (such as reduced swimming activity, reduced light-phase activity per bout, increased sleep time and delayed activity peak) were also present in older (4–6 dpf) larvae exposed to BPA, in addition to the reduced tail coiling frequency. Similarly to the tail coiling data, such locomotor activity deficiencies are consistent with ALS, and their characterisation is critical for the selection of relevant endpoints in preclinical drug development studies. However, locomotor deficits can arise by several different mechanisms, including bioenergetic or metabolic disruptions [172], muscle damage [173], or (most meaningfully for ALS modelling) motor neuron dysfunction [111]. Given the pleiotropic nature of BPA toxicity described in the literature [122], a combination of different mechanisms is likely to be responsible for zebrafish hypoactivity. Further work, designed to study the impact of each BPA toxicity mechanism on locomotor activity, can help to clarify its relevance to ALS.

### 3.4.2. Motor neuron morphology

Motor neuron axonopathy, including morphological abnormalities and reduced length, is a common phenotype in zebrafish models of ALS [60, 61, 78, 110, 111] and has been shown to correlate with neurodegeneration and impaired motility [22, 174]. Pronounced axonopathy would contribute to the face validity of sALS models. Previous studies have reported shorter motor neurons in BPA-treated zebrafish [22, 175]. In our preliminary findings, the shortening was not statistically significant, which could be a false negative; thus requiring a larger sample size for clarification. On the other hand, zebrafish exposed to the highest BMAA concentration had *longer* motor neurons than the corresponding controls. The discrepancy between our preliminary results with 100–1000  $\mu$ M BMAA and 3 dpf larvae and previous reports of BMAA-induced axonopathy (5–25  $\mu$ g/L, at 30 hpf) [20] will also require clarification through a larger sample size. Though beyond the

scope of this study, describing the effect of BPA or BMAA exposure in motor neuron over the course of zebrafish ontogeny may provide insights into their toxicity mechanisms. Lastly, since zebrafish have morphologically distinct primary and secondary motor neuron populations [14], further research on sALS models should include studies of each of them in isolation.

### 3.4.3. Oxidative damage and protein quantification

The pathophysiology of ALS includes increased oxidative stress, mitochondrial dysfunction, and loss of proteostasis [3, 39]. Using an immunodetection approach, we found that BPA increases the oxidative damage marker 4-HNE [94]. This is consistent with the known pro-oxidative effects of BPA in cells [131, 132] and model animals [23, 133, 134], and with observations in sALS patients [176]. Additionally, the observed increase in absolute Hsp60 levels, but not in the Hsp60/Sdha ratio, hints that the Hsp60 elevation may be caused by increased mitochondrial mass in BPA-treated zebrafish. Since previous studies showed that BPA impairs the activity of mitochondrial complex enzymes [135], we hypothesise that BPA-induced mitochondrial dysfunction causes increased ROS production and, through compensation feedback mechanisms, a mitochondrial mass increase. An increase in the levels of the mitochondrial proteins Grp75 and Sdha in BPA-exposed larvae would be consistent with this hypothesis and requires further study. The mechanism through which BPA increases oxidative damage and mitochondrial proteins also warrants further study, given the pivotal and early involvement of mitochondrial dysfunction in ALS [177], and the potential relevance for sALS modelling of inducing it with BPA.

Regarding the effect of BPA on proteostasis, we found no difference in the levels of Hsp70, ubiquitylated proteins, and LC3. This suggests that BPA does not induce a generalised stress response with protein degradation and autophagy, while also not mimicking the depletion in the levels of heat shock proteins that happens in sALS patients [178]. Interestingly, our data suggests an increase in the levels of Grp78 – the master regulator of the unfolded protein response in the ER [163] – in larvae exposed to BPA, which is in line with the recent observation that GRP78 is upregulated and directly binds to aggregates in ALS patients [179], thus prompting further clarification of the impact of BPA on ER stress and proteostasis.

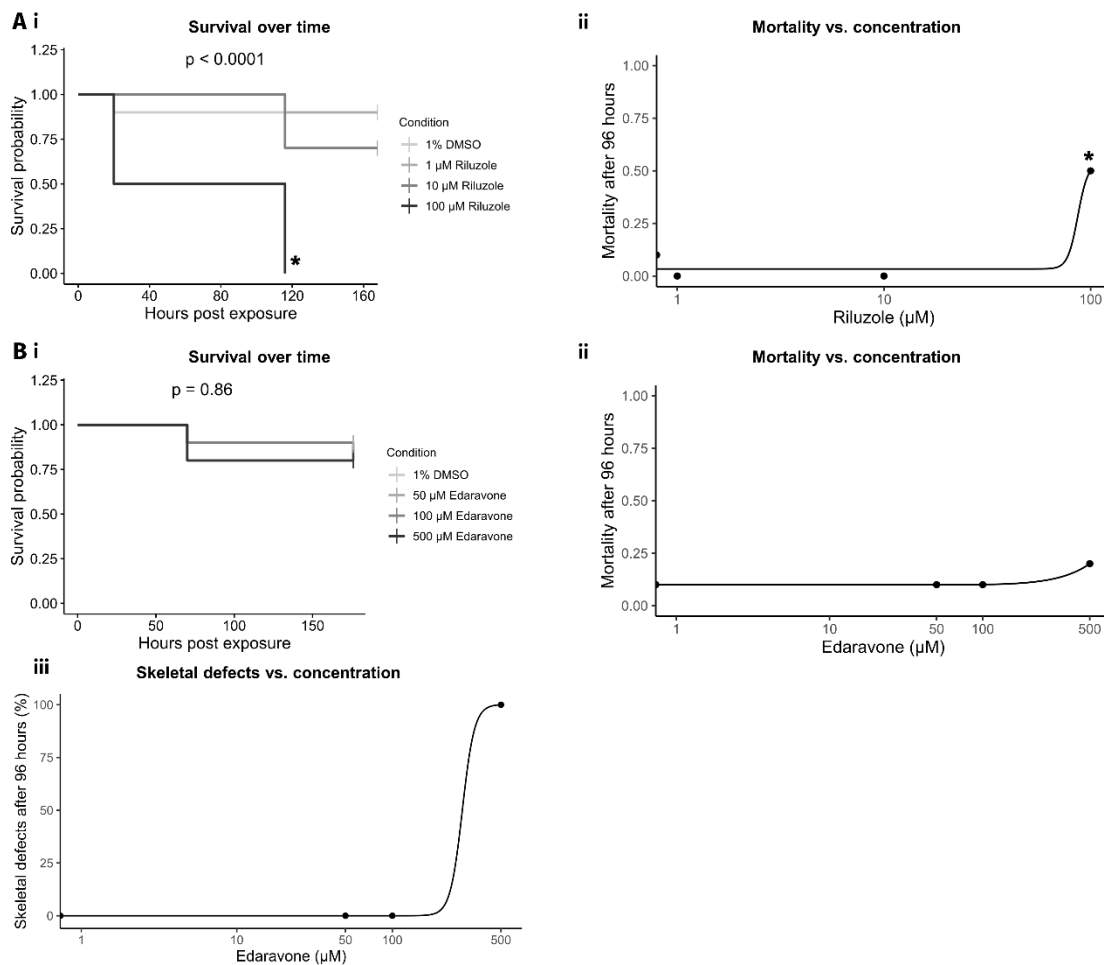
### 3.4.4. Effects of approved ALS drugs on the BPA model of sALS

Currently, riluzole and edaravone are the only FDA-approved drugs for ALS treatment. By scavenging ROS such as  $\text{H}_2\text{O}_2$  and  $\text{ONOO}^-$ , edaravone protects cells against the oxidative damage typically present in ALS patients, while riluzole is thought to act by inhibiting excitotoxicity [112]. We hypothesised that, by evaluating whether these drugs rescued the BPA-induced hypoactivity, we could assess the predictive validity of this model. We found that larvae exposed to BPA in combination with edaravone had higher tail coiling activity than those exposed to BPA alone. Since edaravone is an antioxidant and BPA causes oxidative damage, edaravone is likely to limit the toxicity of BPA by reducing oxidative stress. This possibility should be more thoroughly investigated, specifically regarding the effect on oxidative damage markers (such as 4-HNE) of the combination of edaravone with BPA. Given the links between redox and mitochondrial dysfunctions observed in ALS and the identical dysfunctions seen in BPA-treated zebrafish, the study of this zebrafish model may provide insights into the mechanism(s) of action of edaravone. On the other hand, our current results do not support the hypothesis that  $0.5\ \mu\text{M}$  riluzole can rescue BPA-induced hypoactivity, which suggests that excitotoxicity is not a primary mechanism of BPA toxicity. Thus, the predictive validity of the BPA model of sALS for drug development is likely limited to drugs targeting the mitochondrial and redox component of the disease.

### 3.5. Conclusions

This study expands the characterisation of the previously proposed BPA- and BMAA-induced zebrafish models of sALS. It supports the utility of these models for fundamental study and development of drugs targeting specific pathophysiological pathways of ALS. Both BPA and BMAA cause ALS-consistent motor phenotypes in zebrafish, while the mechanisms through which they do so have varying degrees of relevance for ALS. Though BPA exposure is known to also cause unspecific toxicity, we found that it induces oxidative stress and mitochondrial dysfunction, similarly to clinical findings in ALS patients. Thus, it is a promising model for the redox/mitochondrial component of sALS physiopathology. On the other hand, BMAA exposure is known to induce excitotoxicity, which is relevant for ALS, though the motor phenotypes it causes in zebrafish are more limited than BPA's. This study also highlights the potential utility of zebrafish tail coiling, locomotor activity and sleep parameters as endpoints in the study and preclinical development of new ALS therapies. Overall, no animal model is universal, and the characterisation of the effects of BPA and BMAA in zebrafish should assist the selection of adequate models and assays in the development of therapies for sALS.

### 3.6. Supplemental figures



**Supplemental Fig. 1 – The riluzole and edaravone solutions are bioactive.** (A) Effect of riluzole on zebrafish survival: (i) Kaplan-Meier survival representation of zebrafish larvae exposed to selected riluzole concentrations,  $p$  value on graph from the global log-rank test; (ii) mortality after 96 hours of exposure, per riluzole concentration;  $n = 10$  larvae; \*  $p < .05$  vs. solvent control, Cox's proportional hazards regression. (B) Effect of edaravone on zebrafish survival: (i) Kaplan-Meier survival representation of zebrafish larvae exposed to selected edaravone concentrations,  $p$  value on graph from the global log-rank test; (ii) mortality after 96 hours of exposure, per edaravone concentration; Cox's proportional hazards regression; (iii) percentage of larvae with skeletal defects after 96 hours, per edaravone concentration;  $n = 10$  larvae.





## 4. General discussion and conclusions

In this dissertation, we aimed to review and advance the state of the art of ALS modelling in zebrafish. The first main objective, the **integration and review of knowledge regarding model generation, model phenotypes and existing models**, was addressed in Chapter 2. In it, we describe the abundance and variety of the existing ALS models in zebrafish. Zebrafish models of ALS can be generated using a wealth of genetic modifications or chemical exposures, and many phenotypic endpoints have been used in their characterisation. The diversity of described ALS-relevant phenotypes, however, means that the description of every model is different, complicating comparisons. The translational utility of zebrafish models hinges on understanding the ALS features that each model mimics, and how well it reflects the pathophysiology of different patient subsets (such as idiopathic sALS patients, C9ORF72-ALS patients, FUS-ALS patients, etc.). Thus, wide-scope characterisation and systematisation of existing zebrafish models is needed. There are also important research opportunities on the use of recently proposed genetic targets and toxin combinations for the generation of new models.

The second main objective of this dissertation, the **characterisation of the face validity of chemically induced zebrafish models of sALS**, answers the need (identified in chapter 2) to understand the ALS features that each model mimics. In chapter 3, we described behavioural, motor neuron, and molecular phenotypes of the BPA- and BMAA-induced models. Both models mimic some features of ALS, while presenting some important limitations. BPA-exposed zebrafish showed robust behavioural phenotypes (overall hypoactivity, especially during the light phase of the day), some ALS-consistent molecular changes (such as increased oxidative stress and mitochondrial dysfunction), and no statistically significant changes in motor axon length or morphology. On the other hand, BMAA-exposed zebrafish show behavioural phenotypes, specifically with a reduction in embryo tail coiling activity.

The third main objective, the **characterisation of the predictive validity of chemically induced zebrafish models of sALS**, was also addressed in chapter 3. We evaluated the capacity of the FDA-approved ALS drugs edaravone and riluzole to rescue BPA-induced behavioural phenotypes in zebrafish. Our results suggest that edaravone can at least partially rescue the BPA-induced model, supporting its predictive validity for the development of drugs targeting ALS-related oxidative stress. This kind of research

can also help the characterisation of the toxicity mechanisms underlying the observed phenotypes: in this case, the rescue of BPA-induced hypoactivity with edaravone suggests that oxidative stress is one of the main mechanisms that lead to that hypoactivity.

**Future work** should focus on standardising the characterisation of existing zebrafish models of ALS, developing new and more relevant models, and applying existing models to translational research. Regarding the BPA-induced model, future research should try to elucidate the parallels between the physiopathology of ALS and the oxidative, mitochondrial and ER toxicity of BPA. Finally, the mechanisms through which BMAA induces behavioural phenotypes in zebrafish, as well as the predictive validity of the BMAA-induced zebrafish model, should be clarified.

Overall, zebrafish models of ALS (and, specifically, the BPA and BMAA sALS models) are promising tools in ALS research, if their underlying mechanisms and scope of applicability are well understood and considered. With this dissertation, we reviewed previously disperse state-of-the-art information on several aspects of modelling ALS in zebrafish and advanced the existing knowledge regarding two chemically induced models, which may play a role on the research and development of future therapies for ALS.





## 5. References

1. Griffiths L. *How to Draw a Zebrafish*. 2013 [cited 2022 Sep. 10]; Available from: <http://zebrafishart.blogspot.com/2013/05/heres-new-drawing-this-time-of-baby.html>.
2. Albom M, *Tuesdays with Morrie*. 1997: Warner.
3. Hardiman O, Al-Chalabi A, Chio A, Corr EM, Logroscino G, Robberecht W, . . . van den Berg LH (2017) Amyotrophic lateral sclerosis. *Nature Reviews Disease Primers* 3(1):17071. <https://doi.org/10.1038/nrdp.2017.71>
4. Niedermeyer S, Murn M, Choi PJ (2019) Respiratory Failure in Amyotrophic Lateral Sclerosis. *Chest* 155(2):401-408. <https://doi.org/10.1016/j.chest.2018.06.035>
5. Achtert K, Kerkemeyer L (2021) The economic burden of amyotrophic lateral sclerosis: a systematic review. *The European Journal of Health Economics* 22(8):1151-1166. <https://doi.org/10.1007/s10198-021-01328-7>
6. Al-Chalabi A, Hardiman O, Kiernan MC, Chiò A, Rix-Brooks B, van den Berg LH (2016) Amyotrophic lateral sclerosis: moving towards a new classification system. *The Lancet Neurology* 15(11):1182-1194. [https://doi.org/10.1016/S1474-4422\(16\)30199-5](https://doi.org/10.1016/S1474-4422(16)30199-5)
7. Al-Chalabi A, Hardiman O (2013) The epidemiology of ALS: a conspiracy of genes, environment and time. *Nature Reviews Neurology* 9(11):617-628. <https://doi.org/10.1038/nrneurol.2013.203>
8. Katzung BG, Trevor AJ (2012) *Basic & clinical pharmacology*.
9. Patton EE, Zon LI, Langenau DM (2021) Zebrafish disease models in drug discovery: from preclinical modelling to clinical trials. *Nature Reviews Drug Discovery* 20(8):611-628. <https://doi.org/10.1038/s41573-021-00210-8>
10. Lieschke GJ, Currie PD (2007) Animal models of human disease: zebrafish swim into view. *Nature Reviews Genetics* 8(5):353-367. <https://doi.org/10.1038/nrg2091>
11. Wang X, Zhang JB, He KJ, Wang F, Liu CF (2021) Advances of Zebrafish in Neurodegenerative Disease: From Models to Drug Discovery. *Frontiers in Pharmacology* 12. <https://doi.org/10.3389/fphar.2021.713963>
12. Kalueff AV, Gebhardt M, Stewart AM, Cachat JM, Brimmer M, Chawla JS, . . . Schneider H (2013) Towards a comprehensive catalog of zebrafish behavior 1.0 and beyond. *Zebrafish* 10(1):70-86. <https://doi.org/10.1089/zeb.2012.0861>
13. Cassar S, Adatto I, Freeman JL, Gamse JT, Iturria I, Lawrence C, . . . Zon LI (2020) Use of Zebrafish in Drug Discovery Toxicology. *Chemical Research in Toxicology* 33(1):95-118. <https://doi.org/10.1021/acs.chemrestox.9b00335>
14. Babin PJ, Goizet C, Raldúa D (2014) Zebrafish models of human motor neuron diseases: Advantages and limitations. *Progress in Neurobiology* 118:36-58. <https://doi.org/10.1016/j.pneurobio.2014.03.001>
15. Xi Y, Noble S, Ekker M (2011) Modeling neurodegeneration in zebrafish. *Current Neurology and Neuroscience Reports* 11(3):274-282. <https://doi.org/10.1007/s11910-011-0182-2>
16. Bandmann O, Burton EA (2010) Genetic zebrafish models of neurodegenerative diseases. *Neurobiology of Disease* 40(1):58-65. <https://doi.org/10.1016/j.nbd.2010.05.017>

17. Howe K, Clark MD, Torroja CF, Torrance J, Berthelot C, Muffato M, . . . Stemple DL (2013) The zebrafish reference genome sequence and its relationship to the human genome. *Nature* 496(7446):498-503. <https://doi.org/10.1038/nature12111>
18. Braems E, Tziortzouda P, Van Den Bosch L (2021) Exploring the alternative: Fish, flies and worms as preclinical models for ALS. *Neuroscience Letters* 759. <https://doi.org/10.1016/j.neulet.2021.136041>
19. Purdie EL, Metcalf JS, Kashmiri S, Codd GA (2009) Toxicity of the cyanobacterial neurotoxin  $\beta$ -N-methylamino-L-alanine to three aquatic animal species. *Amyotrophic Lateral Sclerosis* 10(SUPPL. 2):67-70. <https://doi.org/10.3109/17482960903273551>
20. Powers S, Kwok S, Lovejoy E, Lavin T, Sher RB (2017) Embryonic exposure to the environmental neurotoxin BMAA negatively impacts early neuronal development and progression of neurodegeneration in the Sod1-G93R zebrafish model of amyotrophic lateral sclerosis. *Toxicological Sciences* 157(1):129-140. <https://doi.org/10.1093/toxsci/kfx020>
21. Roy U, Conklin L, Schiller J, Matysik J, Berry JP, Alia A (2017) Metabolic profiling of zebrafish (*Danio rerio*) embryos by NMR spectroscopy reveals multifaceted toxicity of  $\beta$ -methylamino-L-alanine (BMAA). *Scientific Reports* 7(1). <https://doi.org/10.1038/s41598-017-17409-8>
22. Morrice JR, Gregory-Evans CY, Shaw CA (2018) Modeling Environmentally-Induced Motor Neuron Degeneration in Zebrafish. *Scientific reports* 8(1):4890-4890. <https://doi.org/10.1038/s41598-018-23018-w>
23. Wang X, Dong Q, Chen Y, Jiang H, Xiao Q, Wang Y, . . . Yang D (2013) Bisphenol A affects axonal growth, musculature and motor behavior in developing zebrafish. *Aquatic Toxicology* 142-143:104-113. <https://doi.org/10.1016/j.aquatox.2013.07.011>
24. Gois AM, Mendonça DMF, Freire MAM, Santos JR (2020) IN VITRO AND IN VIVO MODELS OF AMYOTROPHIC LATERAL SCLEROSIS: AN UPDATED OVERVIEW. *Brain Research Bulletin* 159:32-43. <https://doi.org/10.1016/j.brainresbull.2020.03.012>
25. Ryan M, Heverin M, McLaughlin RL, Hardiman O (2019) Lifetime Risk and Heritability of Amyotrophic Lateral Sclerosis. *JAMA Neurol* 76(11):1367-1374. <https://doi.org/10.1001/jamaneurol.2019.2044>
26. Lacomblez L, Bensimon G, Leigh PN, Guillet P, Meininger V (1996) Dose-ranging study of riluzole in amyotrophic lateral sclerosis. Amyotrophic Lateral Sclerosis/Riluzole Study Group II. *Lancet* 347(9013):1425-31. [https://doi.org/10.1016/s0140-6736\(96\)91680-3](https://doi.org/10.1016/s0140-6736(96)91680-3)
27. Writing Group on behalf of the Edaravone (MCI-186) ALS 19 Study Group. (2017) Safety and efficacy of edaravone in well defined patients with amyotrophic lateral sclerosis: a randomised, double-blind, placebo-controlled trial. *Lancet Neurol* 16(7):505-512. [https://doi.org/10.1016/s1474-4422\(17\)30115-1](https://doi.org/10.1016/s1474-4422(17)30115-1)
28. Shefner J, Heiman-Patterson T, Pioro EP, Wiedau-Pazos M, Liu S, Zhang J, . . . Apple S (2020) Long-term edaravone efficacy in amyotrophic lateral sclerosis: Post-hoc analyses of Study 19 (MCI186-19). *Muscle Nerve* 61(2):218-221. <https://doi.org/10.1002/mus.26740>
29. Phukan J, Elamin M, Bede P, Jordan N, Gallagher L, Byrne S, . . . Hardiman O (2012) The syndrome of cognitive impairment in amyotrophic lateral sclerosis: a population-based study. *J Neurol Neurosurg Psychiatry* 83(1):102-8. <https://doi.org/10.1136/jnnp-2011-300188>
30. Liu S, Huang Y, Tai H, Zhang K, Wang Z, Shen D, . . . Ding Q (2018) Excessive daytime sleepiness in Chinese patients with sporadic amyotrophic lateral sclerosis and its association with cognitive and behavioural impairments. *Journal of Neurology, Neurosurgery & Psychiatry* 89(10):1038-1043. <https://doi.org/10.1136/jnnp-2018-318810>

31. Diaz-Abad M, Buczyner JR, Venza BR, Scharf SM, Kwan JY, Lubinski B, Russell JW (2018) Poor sleep quality in patients with amyotrophic lateral sclerosis at the time of diagnosis. *Journal of clinical neuromuscular disease* 20(2):60-68. <https://doi.org/10.1097/CND.000000000000234>
32. Coco DL, Mattaliano P, Spataro R, Mattaliano A, La Bella V (2011) Sleep–wake disturbances in patients with amyotrophic lateral sclerosis. *Journal of Neurology, Neurosurgery & Psychiatry* 82(8):839-842. <https://doi.org/10.1136/jnnp.2010.228007>
33. Ahmed RM, Newcombe RE, Piper AJ, Lewis SJ, Yee BJ, Kiernan MC, Grunstein RR (2016) Sleep disorders and respiratory function in amyotrophic lateral sclerosis. *Sleep medicine reviews* 26:33-42. <https://doi.org/10.1016/j.smrv.2015.05.007>
34. McCarter SJ, St Louis EK, Boeve BF (2016) Sleep Disturbances in Frontotemporal Dementia. *Curr Neurol Neurosci Rep* 16(9):85. <https://doi.org/10.1007/s11910-016-0680-3>
35. McCauley ME, Baloh RH (2019) Inflammation in ALS/FTD pathogenesis. *Acta Neuropathologica* 137(5):715-730. <https://doi.org/10.1007/s00401-018-1933-9>
36. Ling SC, Polymenidou M, Cleveland DW (2013) Converging mechanisms in als and FTD: Disrupted RNA and protein homeostasis. *Neuron* 79(3):416-438. <https://doi.org/10.1016/j.neuron.2013.07.033>
37. Mandrioli J, Mediani L, Alberti S, Carra S (2020) ALS and FTD: Where RNA metabolism meets protein quality control. *Seminars in Cell & Developmental Biology* 99:183-192. <https://doi.org/10.1016/j.semcdb.2019.06.003>
38. Zou Z-Y, Zhou Z-R, Che C-H, Liu C-Y, He R-L, Huang H-P (2017) Genetic epidemiology of amyotrophic lateral sclerosis: a systematic review and meta-analysis. *Journal of Neurology, Neurosurgery & Psychiatry* 88(7):540. <https://doi.org/10.1136/jnnp-2016-315018>
39. Brown RH, Al-Chalabi A (2017) Amyotrophic lateral sclerosis. *New England Journal of Medicine* 377(2):162-172. <https://doi.org/10.1056/NEJMr1603471>
40. de Jong SW, Huisman MHB, Sutedja NA, van der Kooi AJ, de Visser M, Schelhaas HJ, . . . van den Berg LH (2012) Smoking, Alcohol Consumption, and the Risk of Amyotrophic Lateral Sclerosis: A Population-based Study. *American Journal of Epidemiology* 176(3):233-239. <https://doi.org/10.1093/aje/kws015>
41. Beard JD, Engel LS, Richardson DB, Gammon MD, Baird C, Umbach DM, . . . Kamel F (2016) Military service, deployments, and exposures in relation to amyotrophic lateral sclerosis etiology. *Environment International* 91:104-115. <https://doi.org/10.1016/j.envint.2016.02.014>
42. Ingre C, Roos PM, Piehl F, Kamel F, Fang F (2015) Risk factors for amyotrophic lateral sclerosis. *Clinical epidemiology* 7:181-193. <https://doi.org/10.2147/CLEP.S37505>
43. Chiu AS, Gehringer MM, Welch JH, Neilan BA (2011) Does  $\alpha$ -amino- $\beta$ -methylaminopropionic acid (BMAA) play a role in neurodegeneration? *International Journal of Environmental Research and Public Health* 8(9):3728-3746. <https://doi.org/10.3390/ijerph8093728>
44. De Munck E, Muñoz-Sáez E, Miguel BG, Solas MT, Ojeda I, Martínez A, . . . Arahuetes RM (2013)  $\beta$ -N-methylamino-l-alanine causes neurological and pathological phenotypes mimicking Amyotrophic Lateral Sclerosis (ALS): The first step towards an experimental model for sporadic ALS. *Environmental Toxicology and Pharmacology* 36(2):243-255. <https://doi.org/10.1016/j.etap.2013.04.007>
45. Cox PA, Davis DA, Mash DC, Metcalf JS, Banack SA (2016) Dietary exposure to an environmental toxin triggers neurofibrillary tangles and amyloid deposits in the brain.

- Proceedings of the Royal Society B: Biological Sciences 283(1823):20152397.  
<https://doi.org/10.1098/rspb.2015.2397>
46. Li W, Lee M-H, Henderson L, Tyagi R, Bachani M, Steiner J, . . . Johnson K (2015) Human endogenous retrovirus-K contributes to motor neuron disease. *Science translational medicine* 7(307). <https://doi.org/10.1126/scitranslmed.aac8201>
  47. Douville R, Liu J, Rothstein J, Nath A (2011) Identification of active loci of a human endogenous retrovirus in neurons of patients with amyotrophic lateral sclerosis. *Annals of neurology* 69(1):141-151. <https://doi.org/10.1002/ana.22149>
  48. Chernoff N, Hill DJ, Diggs DL, Faison BD, Francis BM, Lang JR, . . . Winnik WM (2017) A critical review of the postulated role of the non-essential amino acid,  $\beta$ -N-methylamino-L-alanine, in neurodegenerative disease in humans. *J Toxicol Environ Health B Crit Rev* 20(4):1-47. <https://doi.org/10.1080/10937404.2017.1297592>
  49. Dunlop RA, Banack SA, Bishop SL, Metcalf JS, Murch SJ, Davis DA, . . . Bradley WG (2021) Is Exposure to BMAA a Risk Factor for Neurodegenerative Diseases? A Response to a Critical Review of the BMAA Hypothesis. *Neurotoxicity Research* 39(1):81-106.  
<https://doi.org/10.1007/s12640-020-00302-0>
  50. Balendra R, Isaacs AM (2018) C9orf72-mediated ALS and FTD: multiple pathways to disease. *Nature Reviews Neurology* 14(9):544-558. <https://doi.org/10.1038/s41582-018-0047-2>
  51. Gurney ME, Pu H, Chiu AY, Canto MCD, Polchow CY, Alexander DD, . . . Siddique T (1994) Motor Neuron Degeneration in Mice that Express a Human Cu,Zn Superoxide Dismutase Mutation. *Science* 264(5166):1772-1775. <https://doi.org/10.1126/science.8209258>
  52. Ito H, Wate R, Zhang J, Ohnishi S, Kaneko S, Ito H, . . . Kusaka H (2008) Treatment with edaravone, initiated at symptom onset, slows motor decline and decreases SOD1 deposition in ALS mice. *Experimental Neurology* 213(2):448-455.  
<https://doi.org/10.1016/j.expneurol.2008.07.017>
  53. Liguori F, Amadio S, Volonté C (2021) Where and Why Modeling Amyotrophic Lateral Sclerosis. *International Journal of Molecular Sciences* 22(8):3977.  
<https://doi.org/10.3390/ijms22083977>
  54. Patten SA, Parker JA, Wen XY, Drapeau P (2016) Simple animal models for amyotrophic lateral sclerosis drug discovery. *Expert Opinion on Drug Discovery* 11(8):797-804.  
<https://doi.org/10.1080/17460441.2016.1196183>
  55. Hiller M, Agarwal S, Notwell J, Parikh R, Guturu H, Wenger A, Bejerano G (2013) Computational methods to detect conserved non-genic elements in phylogenetically isolated genomes: Application to zebrafish. *Nucleic acids research* 41.  
<https://doi.org/10.1093/nar/gkt557>
  56. Braasch I, Postlethwait JH, *Polyploidy in fish and the teleost genome duplication*, in *Polyploidy and genome evolution*. 2012, Springer. p. 341-383.
  57. Becker CG, Becker T, *Zebrafish as a Model System for Successful Spinal Cord Regeneration*, in *Model Organisms in Spinal Cord Regeneration*. 2006. p. 289-319.
  58. Martin RM, Bereman MS, Marsden KC (2021) BMAA and MCLR interact to modulate behavior and exacerbate molecular changes related to neurodegeneration in larval zebrafish. *Toxicological Sciences* 179(2):251-261. <https://doi.org/10.1093/toxsci/kfaa178>
  59. Lam PY, Peterson RT (2019) Developing zebrafish disease models for in vivo small molecule screens. *Current Opinion in Chemical Biology* 50:37-44.  
<https://doi.org/10.1016/j.cbpa.2019.02.005>

60. Lemmens R, Van Hoecke A, Hersmus N, Geelen V, D'Hollander I, Thijs V, . . . Robberecht W (2007) Overexpression of mutant superoxide dismutase 1 causes a motor axonopathy in the zebrafish. *Hum Mol Genet* 16(19):2359-65. <https://doi.org/10.1093/hmg/ddm193>
61. Laird AS, Van Hoecke A, De Muynck L, Timmers M, Van Den Bosch L, Van Damme P, Robberecht W (2010) Progranulin is neurotrophic in vivo and protects against a mutant TDP-43 induced axonopathy. *PloS one* 5(10):e13368. <https://doi.org/10.1371/journal.pone.0013368>
62. Vaccaro A, Patten SA, Aggad D, Julien C, Maios C, Kabashi E, . . . Parker JA (2013) Pharmacological reduction of ER stress protects against TDP-43 neuronal toxicity in vivo. *Neurobiology of disease* 55:64-75. <https://doi.org/10.1016/j.nbd.2013.03.015>
63. Kabashi E, Lin L, Tradewell ML, Dion PA, Bercier V, Bourgouin P, . . . Velde CV (2010) Gain and loss of function of ALS-related mutations of TARDBP (TDP-43) cause motor deficits in vivo. *Human molecular genetics* 19(4):671-683. <https://doi.org/10.1093/hmg/ddp534>
64. Bosco DA, Lemay N, Ko HK, Zhou H, Burke C, Kwiatkowski TJ, Jr., . . . Hayward LJ (2010) Mutant FUS proteins that cause amyotrophic lateral sclerosis incorporate into stress granules. *Human molecular genetics* 19(21):4160-4175. <https://doi.org/10.1093/hmg/ddq335>
65. Yeh TH, Liu HF, Li YW, Lu CS, Shih HY, Chiu CC, . . . Cheng YC (2018) C9orf72 is essential for neurodevelopment and motility mediated by Cyclin G1. *Exp Neurol* 304:114-124. <https://doi.org/10.1016/j.expneurol.2018.03.002>
66. Pei W, Burgess SM, *Microinjection in Zebrafish for Genome Editing and Functional Studies*, in *Microinjection: Methods and Protocols*, C. Liu and Y. Du, Editors. 2019, Springer New York: New York, NY. p. 459-474.
67. Housden BE, Muhar M, Gemberling M, Gersbach CA, Stainier DYR, Seydoux G, . . . Perrimon N (2017) Loss-of-function genetic tools for animal models: cross-species and cross-platform differences. *Nature Reviews Genetics* 18(1):24-40. <https://doi.org/10.1038/nrg.2016.118>
68. Schulte-Merker S, Stainier DYR (2014) Out with the old, in with the new: reassessing morpholino knockdowns in light of genome editing technology. *Development* 141(16):3103-3104. <https://doi.org/10.1242/dev.112003>
69. Kok FO, Shin M, Ni C-W, Gupta A, Grosse AS, van Impel A, . . . Lawson ND (2015) Reverse Genetic Screening Reveals Poor Correlation between Morpholino-Induced and Mutant Phenotypes in Zebrafish. *Developmental Cell* 32(1):97-108. <https://doi.org/10.1016/j.devcel.2014.11.018>
70. Gut P, Reischauer S, Stainier DYR, Arnaout R (2017) Little Fish, Big Data: Zebrafish as a Model for Cardiovascular and Metabolic Disease. *Physiological Reviews* 97(3):889-938. <https://doi.org/10.1152/physrev.00038.2016>
71. Da Costa MMJ, Allen CE, Higginbottom A, Ramesh T, Shaw PJ, McDermott CJ (2014) A new zebrafish model produced by TILLING of SOD1-related amyotrophic lateral sclerosis replicates key features of the disease and represents a tool for in vivo therapeutic screening. *DMM Disease Models and Mechanisms* 7(1):73-81. <https://doi.org/10.1242/dmm.012013>
72. Bourefis AR, Campanari ML, Buee-Scherrer V, Kabashi E (2020) Functional characterization of a FUS mutant zebrafish line as a novel genetic model for ALS. *Neurobiol Dis* 142:104935. <https://doi.org/10.1016/j.nbd.2020.104935>
73. Ramesh T, Lyon AN, Pineda RH, Wang C, Janssen PM, Canan BD, . . . Beattie CE (2010) A genetic model of amyotrophic lateral sclerosis in zebrafish displays phenotypic hallmarks of motoneuron disease. *Dis Model Mech* 3(9-10):652-62. <https://doi.org/10.1242/dmm.005538>

74. Shaw MP, Higginbottom A, McGown A, Castelli LM, James E, Hautbergue GM, . . . Ramesh TM (2018) Stable transgenic C9orf72 zebrafish model key aspects of the ALS/FTD phenotype and reveal novel pathological features. *Acta neuropathologica communications* 6(1):1-16. <https://doi.org/10.1186/s40478-018-0629-7>
75. Cheresiz SV, Volgin AD, Kokorina Evsyukova A, Bashirzade AAO, Demin KA, de Abreu MS, . . . Kalueff AV (2020) Understanding neurobehavioral genetics of zebrafish. *Journal of Neurogenetics* 34(2):203-215. <https://doi.org/10.1080/01677063.2019.1698565>
76. Yeh J-RJ, Munson KM, Elagib KE, Goldfarb AN, Sweetser DA, Peterson RT (2009) Discovering chemical modifiers of oncogene-regulated hematopoietic differentiation. *Nature Chemical Biology* 5(4):236-243. <https://doi.org/10.1038/nchembio.147>
77. Acosta JR, Goldsbury C, Winnick C, Badrock AP, Fraser ST, Laird AS, . . . Cole NJ (2014) Mutant human FUS Is ubiquitously mislocalized and generates persistent stress granules in primary cultured transgenic zebrafish cells. *PLoS One* 9(6):e90572. <https://doi.org/10.1371/journal.pone.0090572>
78. Ohki Y, Wenninger-Weinzierl A, Hruscha A, Asakawa K, Kawakami K, Haass C, . . . Schmid B (2017) Glycine-alanine dipeptide repeat protein contributes to toxicity in a zebrafish model of C9orf72 associated neurodegeneration. *Molecular Neurodegeneration* 12(1):6. <https://doi.org/10.1186/s13024-016-0146-8>
79. Linney E, Hardison NL, Lonze BE, Lyons S, DiNapoli L (1999) Transgene Expression in Zebrafish: A Comparison of Retroviral-Vector and DNA-Injection Approaches. *Developmental Biology* 213(1):207-216. <https://doi.org/10.1006/dbio.1999.9376>
80. Liu C, *Strategies for Designing Transgenic DNA Constructs*, in *Lipoproteins and Cardiovascular Disease: Methods and Protocols*, L.A. Freeman, Editor. 2013, Humana Press: Totowa, NJ. p. 183-201.
81. Akerberg AA, Stewart S, Stankunas K (2014) Spatial and Temporal Control of Transgene Expression in Zebrafish. *PLOS ONE* 9(3):e92217. <https://doi.org/10.1371/journal.pone.0092217>
82. Schmid B, Hruscha A, Hög S, Banzhaf-Strathmann J, Strecker K, van der Zee J, . . . Haass C (2013) Loss of ALS-associated TDP-43 in zebrafish causes muscle degeneration, vascular dysfunction, and reduced motor neuron axon outgrowth. *Proceedings of the National Academy of Sciences* 110(13):4986-4991. <https://doi.org/10.1073/pnas.1218311110>
83. Huang P, Xiao A, Zhou M, Zhu Z, Lin S, Zhang B (2011) Heritable gene targeting in zebrafish using customized TALENs. *Nature Biotechnology* 29(8):699-700. <https://doi.org/10.1038/nbt.1939>
84. Sander JD, Cade L, Khayter C, Reyon D, Peterson RT, Joung JK, Yeh J-RJ (2011) Targeted gene disruption in somatic zebrafish cells using engineered TALENs. *Nature Biotechnology* 29(8):697-698. <https://doi.org/10.1038/nbt.1934>
85. Hwang WY, Fu Y, Reyon D, Maeder ML, Tsai SQ, Sander JD, . . . Joung JK (2013) Efficient genome editing in zebrafish using a CRISPR-Cas system. *Nature Biotechnology* 31(3):227-229. <https://doi.org/10.1038/nbt.2501>
86. Armstrong GAB, Liao M, You Z, Lissouba A, Chen BE, Drapeau P (2016) Homology Directed Knockin of Point Mutations in the Zebrafish *tardbp* and *fus* Genes in ALS Using the CRISPR/Cas9 System. *PLOS ONE* 11(3):e0150188. <https://doi.org/10.1371/journal.pone.0150188>
87. Butti Z, Pan YE, Giacomotto J, Patten SA (2021) Reduced C9orf72 function leads to defective synaptic vesicle release and neuromuscular dysfunction in zebrafish. *Communications biology* 4(1):792. <https://doi.org/10.1038/s42003-021-02302-y>

88. Silva RFO, Pinho BR, Santos MM, Oliveira JMA (2022) Disruptions of circadian rhythms, sleep, and stress responses in zebrafish: New infrared-based activity monitoring assays for toxicity assessment. *Chemosphere* 305:135449. <https://doi.org/10.1016/j.chemosphere.2022.135449>
89. Pinho BR, Reis SD, Hartley RC, Murphy MP, Oliveira JMA (2019) Mitochondrial superoxide generation induces a parkinsonian phenotype in zebrafish and huntingtin aggregation in human cells. *Free Radical Biology and Medicine* 130:318-327. <https://doi.org/10.1016/j.freeradbiomed.2018.10.446>
90. Pu YZ, Liang L, Fu AL, Liu Y, Sun L, Li Q, . . . Zhao BQ (2017) Generation of Alzheimer's Disease Transgenic Zebrafish Expressing Human APP Mutation Under Control of Zebrafish appb Promotor. *Curr Alzheimer Res* 14(6):668-679. <https://doi.org/10.2174/1567205013666161201202000>
91. Nunes ME, Müller TE, Braga MM, Fontana BD, Quadros VA, Marins A, . . . Loro VL (2017) Chronic treatment with paraquat induces brain injury, changes in antioxidant defenses system, and modulates behavioral functions in zebrafish. *Molecular Neurobiology* 54(6):3925-3934. <https://doi.org/10.1007/s12035-016-9919-x>
92. van Bebber F, Paquet D, Hruscha A, Schmid B, Haass C (2010) Methylene blue fails to inhibit Tau and polyglutamine protein dependent toxicity in zebrafish. *Neurobiology of Disease* 39(3):265-271. <https://doi.org/10.1016/j.nbd.2010.03.023>
93. Scherzinger E, Lurz R, Turmaine M, Mangiarini L, Hollenbach B, Hasenbank R, . . . Wanker EE (1997) Huntingtin-Encoded Polyglutamine Expansions Form Amyloid-like Protein Aggregates In Vitro and In Vivo. *Cell* 90(3):549-558. [https://doi.org/10.1016/S0092-8674\(00\)80514-0](https://doi.org/10.1016/S0092-8674(00)80514-0)
94. Murphy MP, Bayir H, Belousov V, Chang CJ, Davies KJ, Davies MJ, . . . Janssen-Heininger Y (2022) Guidelines for measuring reactive oxygen species and oxidative damage in cells and in vivo. *Nature Metabolism* 4(6):651-662. <https://doi.org/10.1038/s42255-022-00591-z>
95. Gage PW, Armstrong CM (1968) Miniature End-plate Currents in Voltage-clamped Muscle Fibre. *Nature* 218(5139):363-365. <https://doi.org/10.1038/218363b0>
96. Sorrells S, Toruno C, Stewart RA, Jette C (2013) Analysis of Apoptosis in Zebrafish Embryos by Whole-mount Immunofluorescence to Detect Activated Caspase 3. *JoVE* (82):e51060. <https://doi.org/10.3791/51060>
97. Furutani-Seiki M, Jiang YJ, Brand M, Heisenberg CP, Houart C, Beuchle D, . . . Nusslein-Volhard C (1996) Neural degeneration mutants in the zebrafish, *Danio rerio*. *Development* 123(1):229-239. <https://doi.org/10.1242/dev.123.1.229>
98. Delic J, Coppey J, Magdelenat H, Coppey-Moisán M (1991) Impossibility of acridine orange intercalation in nuclear DNA of the living cell. *Experimental Cell Research* 194(1):147-153. [https://doi.org/10.1016/0014-4827\(91\)90144-J](https://doi.org/10.1016/0014-4827(91)90144-J)
99. Mozhenok TP, Beliaeva TN, Bulychev AG, Kuznetsova IM, Leont'eva EA, Faddeeva MD (1997) [The effect of biologically active compounds on lysosome fusion with phagosomes and the F-actin content in mouse peritoneal macrophages and on the status of the lysosomal membranes in mouse hepatocytes]. *Tsitologiya* 39(7):552-9.
100. Meléndez C, Muñoz P, Segura-Aguilar J (2019) DT-Diaphorase Prevents Aminochrome-Induced Lysosome Dysfunction in SH-SY5Y Cells. *Neurotoxicity Research* 35(1):255-259. <https://doi.org/10.1007/s12640-018-9953-8>
101. Aubry J-P, Durand I, De Paoli P, Banchereau J (1990) 7-Amino-4-methylcoumarin-3-acetic acid-conjugated streptavidin permits simultaneous flow cytometry analysis of either three cell

- surface antigens or one cell surface antigen as a function of RNA and DNA content. *Journal of Immunological Methods* 128(1):39-49. [https://doi.org/10.1016/0022-1759\(90\)90461-4](https://doi.org/10.1016/0022-1759(90)90461-4)
102. Felsenfeld AL, Walker C, Westerfield M, Kimmel C, Streisinger G (1990) Mutations affecting skeletal muscle myofibril structure in the zebrafish. *Development* 108(3):443-459. <https://doi.org/10.1242/dev.108.3.443>
103. (1998) Genetic Nomenclature Guide. Zebrafish. *Trends in Genetics* 14(11, Supplement 1):S.32-S.33. [https://doi.org/10.1016/S0168-9525\(98\)80016-4](https://doi.org/10.1016/S0168-9525(98)80016-4)
104. (1998) Genetic Nomenclature Guide. Human. *Trends in Genetics* 14(11, Supplement 1):S.44-S.47. [https://doi.org/10.1016/S0168-9525\(98\)80021-8](https://doi.org/10.1016/S0168-9525(98)80021-8)
105. Ghasemi M, Brown RH, Jr. (2018) Genetics of Amyotrophic Lateral Sclerosis. *Cold Spring Harbor perspectives in medicine* 8(5):a024125. <https://doi.org/10.1101/cshperspect.a024125>
106. Zu T, Liu Y, Bañez-Coronel M, Reid T, Pletnikova O, Lewis J, . . . Ranum LPW (2013) RAN proteins and RNA foci from antisense transcripts in C9ORF72 ALS and frontotemporal dementia. *Proceedings of the National Academy of Sciences* 110(51):E4968-E4977. <https://doi.org/10.1073/pnas.1315438110>
107. Ash PEA, Bieniek KF, Gendron TF, Caulfield T, Lin W-L, DeJesus-Hernandez M, . . . Petrucelli L (2013) Unconventional Translation of C9ORF72 GGGGCC Expansion Generates Insoluble Polypeptides Specific to c9FTD/ALS. *Neuron* 77(4):639-646. <https://doi.org/10.1016/j.neuron.2013.02.004>
108. Smeyers J, Banchi E-G, Latouche M (2021) C9ORF72: What It Is, What It Does, and Why It Matters. *Frontiers in Cellular Neuroscience* 15. <https://doi.org/10.3389/fncel.2021.661447>
109. Lee YB, Chen HJ, Peres JN, Gomez-Deza J, Attig J, Stalekar M, . . . Shaw CE (2013) Hexanucleotide repeats in ALS/FTD form length-dependent RNA foci, sequester RNA binding proteins, and are neurotoxic. *Cell Rep* 5(5):1178-86. <https://doi.org/10.1016/j.celrep.2013.10.049>
110. Swinnen B, Bento-Abreu A, Gendron TF, Boeynaems S, Bogaert E, Nuyts R, . . . Robberecht W (2018) A zebrafish model for C9orf72 ALS reveals RNA toxicity as a pathogenic mechanism. *Acta Neuropathol* 135(3):427-443. <https://doi.org/10.1007/s00401-017-1796-5>
111. Swaminathan A, Bouffard M, Liao M, Ryan S, Callister JB, Pickering-Brown SM, . . . Drapeau P (2018) Expression of C9orf72-related dipeptides impairs motor function in a vertebrate model. *Hum Mol Genet* 27(10):1754-1762. <https://doi.org/10.1093/hmg/ddy083>
112. Jaiswal MK (2019) Riluzole and edaravone: A tale of two amyotrophic lateral sclerosis drugs. *Med Res Rev* 39(2):733-748. <https://doi.org/10.1002/med.21528>
113. Ōmura S, Crump A (2014) Ivermectin: panacea for resource-poor communities? *Trends in Parasitology* 30(9):445-455. <https://doi.org/10.1016/j.pt.2014.07.005>
114. Sea K, Sohn SH, Durazo A, Sheng Y, Shaw BF, Cao X, . . . Valentine JS (2015) Insights into the Role of the Unusual Disulfide Bond in Copper-Zinc Superoxide Dismutase\*. *Journal of Biological Chemistry* 290(4):2405-2418. <https://doi.org/10.1074/jbc.M114.588798>
115. McGown A, McDermid JR, Panagiotaki N, Tong H, Al Mashhadi S, Redhead N, . . . Ramesh TM (2013) Early interneuron dysfunction in ALS: Insights from a mutant sod1 zebrafish model. *Annals of Neurology* 73(2):246-258. <https://doi.org/10.1002/ana.23780>
116. Wang W-Y, Pan L, Su SC, Quinn EJ, Sasaki M, Jimenez JC, . . . Tsai L-H (2013) Interaction of FUS and HDAC1 regulates DNA damage response and repair in neurons. *Nature neuroscience* 16(10):1383-1391. <https://doi.org/10.1038/nn.3514>

117. Lebedeva S, de Jesus Domingues AM, Butter F, Ketting RF (2017) Characterization of genetic loss-of-function of Fus in zebrafish. *RNA Biology* 14(1):29-35. <https://doi.org/10.1080/15476286.2016.1256532>
118. Prasad A, Bharathi V, Sivalingam V, Girdhar A, Patel BK (2019) Molecular mechanisms of TDP-43 misfolding and pathology in amyotrophic lateral sclerosis. *Frontiers in Molecular Neuroscience* 12. <https://doi.org/10.3389/fnmol.2019.00025>
119. Hewamadduma CAA, Grierson AJ, Ma TP, Pan L, Moens CB, Ingham PW, . . . Shaw PJ (2013) Tardbp1 splicing rescues motor neuron and axonal development in a mutant tardbp zebrafish. *Human Molecular Genetics* 22(12):2376-2386. <https://doi.org/10.1093/hmg/ddt082>
120. Bonifacino T, Zerbo RA, Balbi M, Torazza C, Frumento G, Fedele E, . . . Milanese M (2021) Nearly 30 Years of Animal Models to Study Amyotrophic Lateral Sclerosis: A Historical Overview and Future Perspectives. *International journal of molecular sciences* 22(22):12236. <https://doi.org/10.3390/ijms222212236>
121. Rochester JR (2013) Bisphenol A and human health: A review of the literature. *Reproductive Toxicology* 42:132-155. <https://doi.org/10.1016/j.reprotox.2013.08.008>
122. Gassman NR (2017) Induction of oxidative stress by bisphenol A and its pleiotropic effects. *Environmental and molecular mutagenesis* 58(2):60-71. <https://doi.org/10.1002/em.22072>
123. Morrice JR, Gregory-Evans CY, Shaw CA (2018) Animal models of amyotrophic lateral sclerosis: A comparison of model validity. *Neural Regeneration Research* 13(12):2050-2054. <https://doi.org/10.4103/1673-5374.241445>
124. Ito T, Ando H, Suzuki T, Ogura T, Hotta K, Imamura Y, . . . Handa H (2010) Identification of a Primary Target of Thalidomide Teratogenicity. *Science* 327(5971):1345-1350. <https://doi.org/10.1126/science.1177319>
125. Fang J (2010) A direct hit for thalidomide. *Nature*. <https://doi.org/10.1038/news.2010.117>
126. Kirola L, Mukherjee A, Mutsuddi M (2022) Recent Updates on the Genetics of Amyotrophic Lateral Sclerosis and Frontotemporal Dementia. *Molecular Neurobiology*. <https://doi.org/10.1007/s12035-022-02934-z>
127. Turnquist C, Horikawa I, Foran E, Major EO, Vojtesek B, Lane DP, . . . Harris CC (2016) p53 isoforms regulate astrocyte-mediated neuroprotection and neurodegeneration. *Cell Death Differ* 23(9):1515-28. <https://doi.org/10.1038/cdd.2016.37>
128. Zhao T, Ye S, Tang Z, Guo L, Ma Z, Zhang Y, . . . Chen J (2021) Loss-of-function of p53 isoform  $\Delta 113$ p53 accelerates brain aging in zebrafish. *Cell Death and Disease* 12(2). <https://doi.org/10.1038/s41419-021-03438-9>
129. Jones AR, Iacoangeli A, Adey BN, Bowles H, Shatunov A, Troakes C, . . . Al-Chalabi A (2021) A HML6 endogenous retrovirus on chromosome 3 is upregulated in amyotrophic lateral sclerosis motor cortex. *Scientific Reports* 11(1):14283. <https://doi.org/10.1038/s41598-021-93742-3>
130. Patten SA, Armstrong GAB, Lissouba A, Kabashi E, Parker JA, Drapeau P (2014) Fishing for causes and cures of motor neuron disorders. *DMM Disease Models and Mechanisms* 7(7):799-809. <https://doi.org/10.1242/dmm.015719>
131. Leem Y-H, Oh S, Kang H-J, Kim J-H, Yoon J, Chang J-S (2017) BPA-toxicity via superoxide anion overload and a deficit in  $\beta$ -catenin signaling in human bone mesenchymal stem cells. *Environmental Toxicology* 32(1):344-352. <https://doi.org/10.1002/tox.22239>

132. Babu S, Uppu S, Claville MO, Uppu RM (2013) Prooxidant actions of bisphenol A (BPA) phenoxyl radicals: implications to BPA-related oxidative stress and toxicity. *Toxicology Mechanisms and Methods* 23(4):273-280. <https://doi.org/10.3109/15376516.2012.753969>
133. Bindhumol V, Chitra KC, Mathur PP (2003) Bisphenol A induces reactive oxygen species generation in the liver of male rats. *Toxicology* 188(2-3):117-124. [https://doi.org/10.1016/S0300-483X\(03\)00056-8](https://doi.org/10.1016/S0300-483X(03)00056-8)
134. Wu M, Xu H, Shen Y, Qiu W, Yang M (2011) Oxidative stress in zebrafish embryos induced by short-term exposure to bisphenol A, nonylphenol, and their mixture. *Environmental Toxicology and Chemistry* 30(10):2335-2341. <https://doi.org/10.1002/etc.634>
135. Khan S, Beigh S, Chaudhari BP, Sharma S, Aliul Hasan Abdi S, Ahmad S, . . . Raisuddin S (2016) Mitochondrial dysfunction induced by Bisphenol A is a factor of its hepatotoxicity in rats. *Environmental Toxicology* 31(12):1922-1934. <https://doi.org/10.1002/tox.22193>
136. Pierozan P, Andersson M, Brandt I, Karlsson O (2018) The environmental neurotoxin  $\beta$ -N-methylamino-L-alanine inhibits melatonin synthesis in primary pinealocytes and a rat model. *J Pineal Res* 65(1):e12488. <https://doi.org/10.1111/jpi.12488>
137. Cold Spring Harbor Laboratory Press (2011) E3 medium (for zebrafish embryos). Cold Spring Harb Protoc. <https://doi.org/10.1101/pdb.rec066449>
138. Edelstein A, Amodaj N, Hoover K, Vale R, Stuurman N (2010) Computer Control of Microscopes Using  $\mu$ Manager. *Current Protocols in Molecular Biology* 14.20.1-14.20.17. <https://doi.org/10.1002/0471142727.mb1420s92>
139. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, . . . Cardona A (2012) Fiji: an open-source platform for biological-image analysis. *Nature Methods* 9(7):676-682. <https://doi.org/10.1038/nmeth.2019>
140. Kurnia KA, Santoso F, Sampurna BP, Audira G, Huang J-C, Chen KH-C, Hsiao C-D (2021) TCMacro: A Simple and Robust ImageJ-Based Method for Automated Measurement of Tail Coiling Activity in Zebrafish. *Biomolecules* 11(8):1133. <https://doi.org/10.3390/biom11081133>
141. Ferreira T, Miura K, Chef B, Eglinger J (2015) Scripts: BAR 1.1.6. <https://doi.org/10.5281/zenodo.28838>
142. Silva RFO, Pinho BR, Monteiro NM, Santos MM, Oliveira J (2022) Automated analysis of activity, sleep, and rhythmic behaviour in various animal species with the Rtivity software. *Scientific Reports* 12(1):1-11. <https://doi.org/10.1038/s41598-022-08195-z>
143. Pinho BR, Reis SD, Guedes-Dias P, Leitão-Rocha A, Quintas C, Valentão P, . . . Oliveira JMA (2016) Pharmacological modulation of HDAC1 and HDAC6 in vivo in a zebrafish model: Therapeutic implications for Parkinson's disease. *Pharmacological Research* 103:328-339. <https://doi.org/10.1016/j.phrs.2015.11.024>
144. Sylvain NJ, Brewster DL, Ali DW (2010) Zebrafish embryos exposed to alcohol undergo abnormal development of motor neurons and muscle fibers. *Neurotoxicol Teratol* 32(4):472-80. <https://doi.org/10.1016/j.ntt.2010.03.001>
145. Carrel TL, McWhorter ML, Workman E, Zhang H, Wolstencroft EC, Lorson C, . . . Beattie CE (2006) Survival Motor Neuron Function in Motor Axons Is Independent of Functions Required for Small Nuclear Ribonucleoprotein Biogenesis. *The Journal of Neuroscience* 26(43):11014. <https://doi.org/10.1523/JNEUROSCI.1637-06.2006>
146. Pinho BR, Duarte AI, Canas PM, Moreira PI, Murphy MP, Oliveira JMA (2020) The interplay between redox signalling and proteostasis in neurodegeneration: In vivo effects of a

- mitochondria-targeted antioxidant in Huntington's disease mice. *Free Radical Biology and Medicine* 146:372-382. <https://doi.org/10.1016/j.freeradbiomed.2019.11.021>
147. R Core Team (2021) R: A Language and Environment for Statistical Computing.
  148. Wickham H, Bryan J (2019) readxl: Read Excel Files.
  149. Wickham H, Averick M, Bryan J, Chang W, McGowan LDa, François R, . . . Yutani H (2019) Welcome to the tidyverse. *Journal of Open Source Software* 4(43):1686. <https://doi.org/10.21105/joss.01686>
  150. Kassambara A (2021) rstatix: Pipe-Friendly Framework for Basic Statistical Tests.
  151. Andri et mult. al S (2021) DescTools: Tools for Descriptive Statistics.
  152. Therneau TM (2021) A Package for Survival Analysis in R.
  153. Therneau TM, Grambsch PM, *Modeling Survival Data: Extending the Cox Model*. 2000, New York: Springer.
  154. Kassambara A, Kosinski M, Biecek P (2021) survminer: Drawing Survival Curves using 'ggplot2'.
  155. Ritz C, Baty F, Streibig JC, Gerhard D (2015) Dose-Response Analysis Using R. *PLOS ONE* 10(e0146021).
  156. Wilke CO (2020) cowplot: Streamlined Plot Theme and Plot Annotations for 'ggplot2'.
  157. Kassambara A (2020) ggpubr: 'ggplot2' Based Publication Ready Plots.
  158. Geissmann Q (2020) ggetho: Visualisation of High-Throughput Behavioural (i.e. Ethomics) Data.
  159. Attali D, Baker C (2022) ggExtra: Add Marginal Histograms to 'ggplot2', and More 'ggplot2' Enhancements.
  160. Saint-Amant L, Drapeau P (2000) Motoneuron Activity Patterns Related to the Earliest Behavior of the Zebrafish Embryo. *The Journal of Neuroscience* 20(11):3964. <https://doi.org/10.1523/JNEUROSCI.20-11-03964.2000>
  161. Zindler F, Beedgen F, Brandt D, Steiner M, Stengel D, Baumann L, Braunbeck T (2019) Analysis of tail coiling activity of zebrafish (*Danio rerio*) embryos allows for the differentiation of neurotoxicants with different modes of action. *Ecotoxicology and Environmental Safety* 186:109754. <https://doi.org/10.1016/j.ecoenv.2019.109754>
  162. Fernández-Fernández MR, Gragera M, Ochoa-Ibarrola L, Quintana-Gallardo L, Valpuesta JM (2017) Hsp70 - a master regulator in protein degradation. *FEBS Lett* 591(17):2648-2660. <https://doi.org/10.1002/1873-3468.12751>
  163. Ibrahim IM, Abdelmalek DH, Elfiky AA (2019) GRP78: A cell's response to stress. *Life Sci* 226:156-163. <https://doi.org/10.1016/j.lfs.2019.04.022>
  164. Tanida I, Ueno T, Kominami E (2008) LC3 and Autophagy. *Methods Mol Biol* 445:77-88. [https://doi.org/10.1007/978-1-59745-157-4\\_4](https://doi.org/10.1007/978-1-59745-157-4_4)
  165. Chapela D, Sousa S, Martins I, Cristóvão AM, Pinto P, Corte-Real S, Saúde L (2019) A zebrafish drug screening platform boosts the discovery of novel therapeutics for spinal cord injury in mammals. *Sci Rep* 9(1):10475. <https://doi.org/10.1038/s41598-019-47006-w>

166. Hong SJ, Im GJ, Chang J, Chae SW, Lee SH, Kwon SY, . . . Choi J (2013) Protective effects of edaravone against cisplatin-induced hair cell damage in zebrafish. *Int J Pediatr Otorhinolaryngol* 77(6):1025-31. <https://doi.org/10.1016/j.ijporl.2013.04.003>
167. Lechtzin N, Cudkowicz ME, de Carvalho M, Genge A, Hardiman O, Mitsumoto H, . . . Andrews JA (2018) Respiratory measures in amyotrophic lateral sclerosis. *Amyotrophic Lateral Sclerosis and Frontotemporal Degeneration* 19(5-6):321-330. <https://doi.org/10.1080/21678421.2018.1452945>
168. Cedarbaum JM, Stambler N, Malta E, Fuller C, Hilt D, Thurmond B, Nakanishi A (1999) The ALSFRS-R: a revised ALS functional rating scale that incorporates assessments of respiratory function. *Journal of the Neurological Sciences* 169(1):13-21. [https://doi.org/10.1016/S0022-510X\(99\)00210-5](https://doi.org/10.1016/S0022-510X(99)00210-5)
169. Luo L, Song Z, Li X, Zeng Y, He J (2019) Efficacy and safety of edaravone in treatment of amyotrophic lateral sclerosis—a systematic review and meta-analysis. *Neurological Sciences* 40(2):235-241. <https://doi.org/10.1007/s10072-018-3653-2>
170. Hartmaier SL, Rhodes T, Cook SF, Schlusser C, Chen C, Han S, . . . Davé S (2022) Qualitative measures that assess functional disability and quality of life in ALS. *Health and Quality of Life Outcomes* 20(1). <https://doi.org/10.1186/s12955-022-01919-9>
171. Ma Y, Liu H, Wu J, Yuan L, Wang Y, Du X, . . . Zhang H (2019) The adverse health effects of bisphenol A and related toxicity mechanisms. *Environ Res* 176:108575. <https://doi.org/10.1016/j.envres.2019.108575>
172. Yan J, Chen S, Zuo Z, He C, Yi M (2020) Graphene oxide quantum dot exposure induces abnormalities in locomotor activities and mechanisms in zebrafish (*Danio rerio*). *Journal of Applied Toxicology* 40(6):794-803. <https://doi.org/10.1002/jat.3944>
173. Chen X, Zheng J, Teng M, Zhang J, Qian L, Duan M, . . . Wang C (2022) Tralopyril affects locomotor activity of zebrafish (*Danio rerio*) by impairing tail muscle tissue, the nervous system, and energy metabolism. *Chemosphere* 286. <https://doi.org/10.1016/j.chemosphere.2021.131866>
174. Robinson KJ, Yuan KC, Don EK, Hogan AL, Winnick CG, Tym MC, . . . Laird AS (2018) Motor Neuron Abnormalities Correlate with Impaired Movement in Zebrafish that Express Mutant Superoxide Dismutase 1. *Zebrafish* 16(1):8-14. <https://doi.org/10.1089/zeb.2018.1588>
175. Gu J, Guo M, Yin X, Huang C, Qian L, Zhou L, . . . Ji G (2022) A systematic comparison of neurotoxicity of bisphenol A and its derivatives in zebrafish. *Science of The Total Environment* 805:150210. <https://doi.org/10.1016/j.scitotenv.2021.150210>
176. Turner MR, Kiernan MC, Leigh PN, Talbot K (2009) Biomarkers in amyotrophic lateral sclerosis. *The Lancet Neurology* 8(1):94-109. [https://doi.org/10.1016/S1474-4422\(08\)70293-X](https://doi.org/10.1016/S1474-4422(08)70293-X)
177. Smith EF, Shaw PJ, De Vos KJ (2019) The role of mitochondria in amyotrophic lateral sclerosis. *Neuroscience Letters* 710. <https://doi.org/10.1016/j.neulet.2017.06.052>
178. Chen H-J, Mitchell JC, Novoselov S, Miller J, Nishimura AL, Scotter EL, . . . Shaw CE (2016) The heat shock response plays an important role in TDP-43 clearance: evidence for dysfunction in amyotrophic lateral sclerosis. *Brain* 139(5):1417-1432. <https://doi.org/10.1093/brain/aww028>
179. François-Moutal L, Scott DD, Ambrose AJ, Zerio CJ, Rodriguez-Sanchez M, Dissanayake K, . . . Khanna M (2022) Heat shock protein Grp78/BiP/HspA5 binds directly to TDP-43 and mitigates toxicity associated with disease pathology. *Sci Rep* 12(1):8140. <https://doi.org/10.1038/s41598-022-12191-8>



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