New zebrafish models of neurodegeneration

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Abstract

In modern biomedicine the increasing need to develop experimental models to further our understanding of disease conditions and delineate innovative treatments has found in the zebrafish (*Danio rerio*) an experimental model, and indeed a valuable asset, to close the gap between *in vitro* and *in vivo* assays. Translation of ideas at a faster pace is vital in the field of neurodegeneration, with the attempt to slow or prevent the dramatic impact on society's welfare being an essential priority. Our research group has pioneered the use of zebrafish to contribute to the quest for faster and improved understanding and treatment of neurodegeneration in concert with, and inspired by, many others who have primed the study of the zebrafish to understand and search for a cure for disorders of the nervous system. Aware of the many advantages this vertebrate model holds, here we present an update on the recent zebrafish models available to study neurodegeneration with the goal of stimulating further interest and increasing the number of diseases and applications for which they can be exploited. We shall do so by citing and commenting on recent break-throughs made possible via zebrafish, highlighting their benefits for the testing of therapeutics and dissecting of disease mechanisms.

Keywords: neurodegenerative; *Danio rerio*; disease model; therapeutic testing; drug testing; mechanism

Introduction

In recent years the utility of zebrafish for disease modelling, mechanistic studies and therapeutic testing has been recognised by increasing numbers of researchers in many fields, but particularly in the field of neurodegeneration, due to the unique advantages of the zebrafish. These include the ability to generate and house large numbers of vertebrates relatively quickly, cheaply and in a small space, their transparency during development, the sophisticated genetic and phenotyping techniques available, their high level of biological relevance to humans, and the identification of developing zebrafish as a 'replacement model' to avoid the use of protected animals in experiments: this has been recently reviewed in detail elsewhere [1-4]. Of particular note is the use of zebrafish to identify the anti-arthritis drug leflunomide as a potential treatment for melanoma, which is now being tested in a Phase I/II clinical trial [3]. This demonstrates a sufficiently close relationship between zebrafish and human biology to support the continued use of zebrafish for compound testing and drug discovery. Indeed, many other studies hold the promise of leading to new drug treatments [1-3, 5].

There are a huge number of neurodegenerative diseases, and zebrafish disease model generation has only scratched the surface, but recent technological innovations in genome editing promise to widen and strengthen inherited disease modelling in zebrafish [4]. Although gene editing has not yet been used to generate zebrafish models of neurodegenerative disease, models created using this new technology should be published in the near future. In this review we shall critically assess the most notable and recently published models, disease mechanisms and therapeutic testing utilising zebrafish (**Table 1**) and endeavour to stimulate further research in this important area. Although overlaps exist between epilepsy and neurodegenerative disease, we will refrain from reviewing zebrafish models of epilepsy in detail here as this disorder has been recently reviewed [1, 6], along with zebrafish models of mitochondrial disease [7, 8], metabolic disease [9] and neurological disorders [1, 10, 11].

Technological advances relating to zebrafish disease modelling

A plethora of methods are now being used to generate disease models in zebrafish, dependent on the aetiology of the disease to be modelled. The zebrafish is highly amenable to manipulation and methods tend to be based on genetic alterations or chemical treatments and can include a combination of both (for extensive examples please refer to other reviews [1, 8-

11]). The discovery of antisense morpholino oligonucleotides (MO) in 2000 revolutionised zebrafish research [12]. Injecting them into the embryo or tissue of interest results in dose-dependent knock-down of the targeted gene, but with widespread use it has become apparent that they often have off-target effects and several methods are required to show these are not occurring. Mutants generated in mutagenesis screens have a similar issue in that a mutant line may harbour additional, unidentified mutations that could affect phenotypes. These can be limited by several generations of outcrossing. A more effective way to limit this is to use a mutagenesis method that results in few off-target mutations, as offered by recently published gene editing methods such as Zinc Finger Nucleases, TALENS and CRISPR/Cas9 [4]. Of these, CRISPR/Cas9 seems to be the most useful as it is easy to design reagents for any gene target, easy to implement, has a high rate of mutagenesis and a low level of off-target effects. Although initially developed to make mutant zebrafish, TALEN and CRISPR genome editing has now been used to knock-in genes [13], and further development of this technology promises to enable the replacement of zebrafish genes with their human counterparts, likely making the subsequent research using those strains more directly relevant to human disease.

Zebrafish models of neurodegeneration

Neurodegenerative disease has many different causes, ranging from toxic levels of metals to mitochondrial dysfunction or inherited mutations. Here attention will be focused on the recent generation of novel inherited models and transient models. When a mutation causing a human disease is identified, modelling of that disease in zebrafish is often used to provide additional evidence supporting the causative gene. In other cases, zebrafish models have been made as a tool to perform research into finding treatments, often for incurable fatal diseases. As the embryo and larva is transparent and amenable to many more techniques than older zebrafish (e.g. genetic manipulation, transplantation, laser ablation), chemical treatments are cheaper on small fish, and results are achieved faster, it can be advantageous if a disease model has phenotypes at these early developmental stages. Some zebrafish disease models naturally have such an early onset and this reflects the human condition (e.g. Pontocerebellar hypoplasia [14, 15]), others have an early onset even though the human disease onset appears to be later (e.g. CLN2 disease [16]), and yet others are created specifically to have a phenotype during development even though the human disease is adult onset (e.g. Parkinson's Disease [17, 18]). In all cases, the degree to which the model reflects the biology of the human disease must be

taken into account when interpreting the results, in addition to any other species-specific differences.

Pontocerebellar hypoplasia

Pontocerebellar hypoplasia is a group of inherited progressive neurodegenerative disorders with prenatal onset that manifests with microcephaly in infancy or early childhood and fatality in early life [19]. Children have profound intellectual disability and delayed or absent psychomotor skills, accompanied by lack of development of the pons and cerebellum. Several genes, including *TSEN54*, *RARS* and *CLP1* have been identified as harbouring causative mutations with autosomal recessive inheritance [19]. The zebrafish has been used twice to support gene identification. In 2001, Kasher et al. used *tsen54* antisense morpholino (MO) injection in zebrafish to attempt to mimic the human disease phenotype which is caused by mutation in TSEN54, a member of the tRNA splicing endonuclease complex (TSEN) (**Table 1**) [14]. Morpholinos (MO) directed to the ATG or the exon 8 splice donor site gave similar phenotypes: an indistinct mid-hindbrain boundary (which develops into the cerebellum) and a greyness in the brain indicating cell death. The ATG MO was partially rescued by injection of human mRNA and this was taken as an indication that the human and zebrafish genes are not completely interchangeable in terms of function, but that the human gene can provide some of the zebrafish gene function. It is notoriously difficult to interpret this type of rescue experiment, as it remains possible that the phenotypes not rescued by the human mRNA are caused by off-target morpholino toxicity. The authors included survival analysis from a mutant line to corroborate the morpholino results, but without demonstrating that the 24 hpf brain phenotype in the mutant is the same as that in the morphant, the question remains whether the cell death in the brain is attributable to loss of *tsen54* or off-target morpholino toxicity. Such a study highlights the importance of using more than one method to model a disease in zebrafish and to validate the resulting disease phenotypes. One further way to provide corroborating evidence that the phenotype is specific to the gene knockdown is to knockdown other genes known to cause the same disease and compare phenotypes. Kasher et al. did this successfully when they showed essentially the same phenotype is caused by *rars* knockdown in zebrafish by morpholino injection (**Table 1**) [14].

The Kasher et al. study was followed by Schaffer et al, who determined that homozygous mutations in CLP1 can also cause pontocerebellar hypoplasia [15]. CLP1 is another member

of the tRNA splicing endonuclease complex (TSEN) and patient cells were shown to have reduced tRNA splicing activity suggesting a loss-of-function mutation. Schaffer et al. used R44X loss-of-function mutant zebrafish and demonstrated lack of *clp1* mRNA expression, a reduction in expression of the *otx2* midbrain marker and an increase in midbrain and hindbrain TUNEL stain which indicates cell death, both of which had an onset of about 48 hpf (**Table 1**) [15]. As predicted, abnormal spinal motor neurons were demonstrated in the $clp1^{R44X}$ mutants, using SV2 immunohistochemistry as a marker. Visible phenotypes included a curved body, small abnormal head and small eyes, and premature death by 5 dpf. These phenotypes were also seen in the second allele they examined, clp^{L35R} (**Table 1**) [15], and have been demonstrated in other zebrafish models of pontocerebellar hypoplasia (*tsen54* and *rars* morphants described above [14]) and other forms of neurodegeneration (e.g. CLN2 disease) [16]. The curved body and reduction in *otx2* expression was partially rescued by injection of human *CLP1* MRNA but not when it contained the mutation found in patients, showing that function was at least partially conserved and further corroborating the hypothesis that the zebrafish mutation is loss-of-function. Because the authors compared rescue using both normal and mutant human mRNA, it is possible to attribute the partial rescue to the human mRNA, but it remains unknown whether the lack of complete rescue is due to differences between the function of the human and zebrafish proteins or due to the global expression of the human protein causing additional phenotypes - background mutations are unlikely to contribute as several outcrosses were performed [15].

Experiments where p53 was reduced using antisense morpholino injection into clp^{R44X} embryos showed that *otx2* expression was partially rescued and therefore p53 at least partly mediates the phenotype [15]. Given the role of p53 in stress-induced cell death, it was presumed, but not yet demonstrated that this was via a reduction in cell loss. One limitation in the publication was the absence of a hindbrain marker for their analyses, which would have been more relevant than a midbrain marker, given that pontocerebellar hypoplasia predominantly affects development and maintenance of structures that arise from the hindbrain. Even so, this series of experiments highlights very nicely how the zebrafish model can be used to support identification of human disease mutations and probe the underlying mechanisms.

CLN2 disease

The recent publication by our group of a zebrafish model of the autosomal recessive lysosomal storage disorder CLN2 disease [16] illustrates that inherited diseases with an apparent onset in childhood can cause phenotypes during development of the zebrafish, perhaps making it more difficult to justify the direct relevance of the zebrafish model to the disease. CLN2 disease has an onset of around 2 years old and death usually occurs before adolescence [20], yet the zebrafish model has visible phenotypes from 2 dpf (smaller brain and eye, curved body and larger yolk) and dies by 7 dpf [16]. This early onset may be due to the precocious zebrafish development, the fact that it develops ex-ovo, or because TPP1 expression is upregulated earlier in the zebrafish than in humans. In spite of this, for all pathological and clinical signs examined to date, the zebrafish model has phenotypes equivalent to that of patients [16]. CLN2 disease is caused by loss-of-function of the Tri-peptidyl peptidase 1 gene (Tpp1). We examined two *tpp1* mutants and two *tpp1* morphants in zebrafish and demonstrated that strong loss-of-function (homozygous $tpp1^{sa0011}$) resulted in early lethality whereas a weak allele (homozygous $tpp1^{hu3587}$) gave similar phenotypes but at a lower frequency and with a later onset. Compound heterozygotes ($tpp1^{sa0011}/tpp1^{hu3587}$) had an intermediate phenotype (**Table 1**) [16]. Although the compound heterozygotes may appear to be a better model than $tpp1^{sa0011}$ mutants in terms of disease onset, they have a more variable phenotype so far more animals are required for experiments, and the later onset means that many of the advantages for drug discovery and manipulation that the embryo and larvae provide cannot be leveraged.

Hence we focussed on the homozygous $tpp1^{sa0011}$ mutants and were able to show many relevant phenotypes considered to be hallmarks of the disease apart from the reduced lifespan already mentioned: these include seizure-like movements followed by later lack of ability to move, nervous system cell death which was particularly evident in the retina, optic tectum, and cerebellum, reduced myelin, astrocytosis, enlarged lysosomes, and appropriate storage material [16]. As no one has yet demonstrated overt seizures or retinal degeneration in the mouse model [21], this zebrafish model is potentially more relevant to the disease than the mouse model. Compared to disease onset at 7 weeks in the mouse model [21], the embryonic onset and fast progression in the zebrafish model offers the potential to complete experiments much more quickly.

We went on to demonstrate that axon path-finding and secondary proliferation were also impaired in $tpp1^{sa0011}$ mutants [16] and this may be relevant to future therapeutic studies. For example, the lack of proliferation may be because cells need TPP1 to proliferate or because the degenerating brain does not support proliferation of cells. Mosaic analysis could be used to

distinguish between these two possibilities, as was demonstrated by mosaic analysis of the zebrafish *flotte lotte* mutant [22]. If it is shown that the degenerating brain does not support cell proliferation, this would have a large impact on when stem cell therapies would need to be given i.e. before the brain loses the ability to support proliferation. Our research also demonstrated that with careful analysis of locomotion at different stages, seizure-like locomotion can be identified even in a model of a disease with profound motor deficits (**Figure 1 A**) [16]. This means that both locomotion phenotypes could be used for drug discovery, potentially enabling the identification of compounds or drugs that benefit specific aspects of the disease.

Parkinson's Disease and Dementia with Lewy Bodies

α-synuclein (aSyn) is one of the main intracellular storage materials both in Parkinson's Disease (PD) and Dementia with Lewy Bodies (DLB), along with rare forms of PD caused by a variety of mutations in SNCA, the gene encoding α -synuclein (aSyn) [23]. Many cell and animal PD models have been generated using aSyn and studying these have shown that axon degeneration occurs before cell death, but the hypothesis that axon degeneration actually leads to cell death was not tested due to limitations of the available models. O'Donnell et al. used the distinct attributes of the developing zebrafish to answer this question. Their clever use of transgenes allowed them to transiently express aSyn in Rohon Beard sensory neurons and label both transgenic and normal Rohon Beard cells simultaneously with different fluorescent proteins (**Table 1**) [17], avoiding the use of tagged proteins that can cause aggregate formation. aSyn expression caused moderate levels of cell death by 3 dpf, axon swelling was noted at 2 dpf, and axon degeneration by 3 dpf, consistent with other models. Careful time-lapse analysis demonstrated that axon swellings always preceded cell death but axon degeneration (fragmentation) did not precede signs of cell death (**Figure 1 B, C**) [17]. Hence, the use of live fluorescent markers in the transparent zebrafish has provided evidence to support the hypothesis that axon swellings, not axon degeneration, precede cell death in aSyn overexpression models of PD. This experiment also highlights that the axonal compartment is more vulnerable than the cell body to aSyn toxicity.

O'Donnell et al. went on to investigate the relationship between Wallerian degeneration and aSyn. They demonstrated that aSyn did not exacerbate Wallerian degeneration (induced by

transection of axons by a laser) but that this axotomy increased the amount of cell death caused by aSyn, suggesting that axon injury aggravates aSyn toxicity. However, expression of WldS, a protein that protects against Wallerian degeneration, did not reduce cell death caused by aSyn even though it had some positive effects in the axon compartment [17].

In addition, the authors investigated mitochondrial transport using a live *cox8:DsRed* transgene and found that aSyn-expressing axons had a higher density of mitochondria, which were abnormally spherical and swollen, suggesting respiratory chain dysfunction. Timelapse movies showed reduced mitochondrial motility, especially in the anterograde direction, at 2 dpf [17] suggesting that it might contribute to cell death and axonopathy. To test this, they expressed PGC-1α, a transcriptional co-activator that has varied roles in mitochondrial biogenesis and ROS detoxification [18], in the same cells as aSyn and found that both cell death and axonopathy were rescued to normal levels [17], which is supported by experiments in mice where PGC-1α protects dopaminergic neurons in a chemical model of PD. Although it is still very unclear how PGC-1 α is protecting against cell death in this system, this research corroborates PGC-1a as a promising target and illustrates the use of genetic manipulation in zebrafish to find therapeutic targets.

Amyotrophic lateral sclerosis

The identification and development of treatments for neurodegenerative diseases is a major reason behind developing zebrafish models. As mentioned before, other diseases are beginning to benefit from this approach. ALS has a complex aetiology with some families identified with ALS-causing mutations in TAR DNA Binding Protein 43 (TDP-43) [24]. To identify compounds that have potential for treating ALS, Vaccaro et al. used zebrafish (**Table 1**) and the nematode (*C. elegans*) expressing mutant TDP-43 (mTDP-43), via transient injection and as an integrated transgenic respectively, to test three neuroprotective agents: riluzole, lithium and methylene blue [25]. In the zebrafish mTDP-43 model, the touch evoked escape response, a read out for motor function, was almost absent, motor axons were shorter and abnormally branched (as demonstrated by znp-1 immunohistochemistry), and oxidative stress was increased (as demonstrated by dihydrofluorescein diacetate fluorescence in live zebrafish). Vaccaro et al. found that methylene blue improved relevant phenotypes in both models, likely through reducing ER stress [25]. This was the first time that *in vivo* phenotypes had been used

for chemical screening for ALS. Subsequently, Vaccaro et al. determined that the ER is the most likely source of the stress in the *C. elegans* model and therefore selected other chemicals thought to act by reducing ER stress (and the ER unfolded protein response) and treated the nematode and fish mTDP-43 models with these [26]. Three agents (salubrinal, guanabenz and phenazine) were found to rescue paralysis, neurodegeneration and oxidative stress, albeit through different parts of the ER stress pathway (**Figure 1 D**) [26]. These results show that the ER unfolded protein response is an important target for therapeutic development, and this has recently been borne out by guanabenz treatment in G93A mtSOD1 transgenic mice, which results in a delay in onset, elongation of the early disease phase, and lengthened survival [27, 28]. This zebrafish research has therefore contributed directly to the identification of guanabenz, an approved drug for hypertension, as a priority drug to pursue for the treatment of ALS.

Conclusions

The examples in this review demonstrate that zebrafish has clearly some unique advantages over other model organisms for modelling neurodegeneration. However, these advantages can also be their weakness: for example an earlier onset in zebrafish than in humans causes us to question their validity but this attribute also means that the model could be used for time-lapse imaging or drug discovery, with far more experiments that can be done more quickly and cheaply than in mammals.

With the advent of genome editing techniques in zebrafish, the frequency with which new models of monogenetic inherited disease are published is predicted to increase significantly as these will be the most straightforward to model and possibly the most easily equated to human disease. Many rare neurodegenerative disease are devastating, they frequently cause neurodegeneration in children, and their study is mainly supported by small charities. Hence, study of these diseases could gain most by exploiting the advantages of the zebrafish model.

Acknowledgments

Thank you to all members of the Claire Russell and Michelangelo Campanella's research group for the on-going support. RMJ is a postdoctoral researcher funded by a research grant to CR and MC from SPARKS children's charity.

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Figure legends

Figure 1. Developing zebrafish offer significant advantages for disease modelling and drug discovery.

(A) Motor deficits in the *tpp1*^{sa0011} mutant zebrafish at 96 hpf (above) are preceded by seizure-like excessive locomotion at 72 hpf (below). Tracks in a 20 minute period are shown in blue/red. Adapted from Mahmood, F., et al., *A zebrafish model of CLN2 disease is deficient in tripeptidyl peptidase 1 and displays progressive neurodegeneration accompanied by a reduction in proliferation.* Brain, 2013. **136**(Pt 5): p. 1488-1507, with permission from Oxford University Press [16].

(B, C) Time-lapse imaging of neurodegeneration in aSyn-expressing neurons. Cells were imaged every 20 minutes beginning 54 hours post-fertilization (hpf). Axons from at least 11 embryos from each group were transected; representative images from aSyn-expressing animals are shown. Time stamps in images are relative to the start of the imaging period. Axonal varicosities were observed (white arrowheads) several hours before cell death. White arrows point to morphological changes indicative of cell death. Inset represents cell body magnified 2×. Asterisk in B indicates separation of the axon from the cell body. Axonal fragmentation (blue arrowheads) usually did not occur before cell death, and was not stereotyped: it did not occur synchronously along the length of the axon, nor in a retrograde direction (yellow arrows point to distal portions of the axon that are still intact). Scale bars: 50 μm. Reproduced under the Creative Commons Attribution Licence from O'Donnell K C et al. Dis. Model. Mech. 2014; 7: 571-582 [17].

(D) Escape response in mTDP-43 expressing zebrafish and with treatment of salubrinal (Sal), guanabenz (Gua) and phenazine (Phe). Reprinted from Vaccaro, A., et al., *Pharmacological reduction of ER stress protects* against TDP-43 neuronal toxicity in vivo. Neurobiology of Disease, 2013. **55**: p. 64-75 [26], with permission from Elsevier.

Table 1. Summary of the key findings attained from the zebrafish models of neurodegenerative disease reviewed in this article.

Human disease onset	Disease	Type of zebrafish model	Outcome summary	Ref.
Prenatal	Pontocerebellar hypoplasia	tsen54 morphant (ATG)	Brain hypoplasia and loss of structural definition inside the brain including cerebellum at 24 hpf. Partially rescued by co-injecting human TSEN54 mRNA	[14]
		tsen54 morphant (exon 8 splice donor site)	Brain hypoplasia and reduced structural definition inside the brain including cerebellum at 24 hpf.	[14]
		$tsen54^{R228X/R228X}$ mutant	Lethal by 9 dpf (with 62% $tsen54^{R228X-/+}$ heterozygotes surviving to 21 dpf).	[14]
		rars2 morphant (ATG)	Brain hypoplasia and reduced structural definition inside the brain including cerebellum at 24 hpf. Partially rescued by co-injecting human rars2 mRNA.	[14]
		rars2 morphant (exon 6 splice donor site)	Brain hypoplasia and reduced structural definition inside the brain including cerebellum at 24 hpf.	[14]
		$clp1^{R44X}$ mutant	Abnormal head shape and curved tail. Small eyes. Abnormal swimming behavior. Premature death by 5 dpf. The average curve height is partially rescued by human CLP1 mRNA	[15]
		$clp1^{L35R}$ mutant	Similar uniform lethality by 5 dpf than $CLP1^{R44X}$	[15]
Juvenile	CLN2 Disease	$tpp1^{sa0011/sa0011}$ mutants	Curved body, small eyes and head, large yolk at 48hpf. No detectable jaw, pericardial oedema at 72hpf. Lethality by 7dpf. Seizure-like movements followed by later lack of ability to move. Nervous system cell death, lack of proliferation, reduced axons.	[16]
		$tpp1^{hu3587/hu3587}$ mutant	Similar phenotypes to $tpp1^{sa0011}$ but at a lower frequency and with a later onset. Some are adult viable.	[16]
		$tpp1^{sa0011/hu3587}$ mutant	Similar phenotypes to $tpp1^{sa0011}$ but with a later onset of 72hpf	
		tpp1 morphant (exon 2 splice acceptor site)	Abnormal phenotype beginning around 48 h post fertilization including increasingly smaller eyes and head, curvature within the body axis, a reduced jaw	[16]
		tpp1 morphant (ATG)	Abnormal phenotype beginning around 48 h post fertilization including increasingly smaller eyes and head, curvature within the body axis, a reduced jaw	[16]
Adult	Parkinson's Disease and Dementia with Lewy Bodies	Transgenic aSyn-2A-GFP transiently expressed in Rohon Beard neurons	Neuron death and axonopathy between 2-3 dpf. Aberrations in mitochondrial morphology, density and motility at 2 dpf	[17]
	Amyotrophic lateral sclerosis	mTDP-43[G48C] mRNA injection	Abnormally shortened and branched motor neuron axonal processes at 2 dpf. Motor deficit. Rescued by methylene blue and other compounds reducing ER stress.	[25, 26]
		mFUS[R21H] mRNA injection	Abnormally shortened and branched motor neuron axonal processes at 2 dpf. Motor deficit. Rescued by methylene blue.	[25]