



## Review

Zebrafish models of Tauopathy<sup>☆</sup>Qing Bai<sup>a,b</sup>, Edward A. Burton<sup>a,b,c,d,e,f,\*</sup><sup>a</sup> Pittsburgh Institute for Neurodegenerative Diseases, University of Pittsburgh School of Medicine, USA<sup>b</sup> Department of Neurology, University of Pittsburgh School of Medicine, USA<sup>c</sup> Department of Microbiology and Molecular Genetics, University of Pittsburgh School of Medicine, USA<sup>d</sup> Department of Neurology, VA Pittsburgh Healthcare System, USA<sup>e</sup> Geriatric Research, Education and Clinical Center, VA Pittsburgh Healthcare System, USA<sup>f</sup> Division of Movement Disorders, University of Pittsburgh Medical Center, USA

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## ABSTRACT

Tauopathies are a group of incurable neurodegenerative diseases, in which loss of neurons is accompanied by intracellular deposition of fibrillar material composed of hyperphosphorylated forms of the microtubule-associated protein Tau. A zebrafish model of Tauopathy could complement existing murine models by providing a platform for genetic and chemical screens, in order to identify novel therapeutic targets and compounds with disease-modifying potential. In addition, Tauopathy zebrafish would be useful for hypothesis-driven experiments, especially those exploiting the potential to deploy *in vivo* imaging modalities. Several considerations, including conservation of specialized neuronal and other cellular populations, and biochemical pathways implicated in disease pathogenesis, suggest that the zebrafish brain is an appropriate setting in which to model these complex disorders. Novel transgenic zebrafish lines expressing wild-type and mutant forms of human Tau in CNS neurons have recently been reported. These studies show evidence that human Tau undergoes disease-relevant changes in zebrafish neurons, including somato-dendritic relocalization, hyperphosphorylation and aggregation. In addition, preliminary evidence suggests that Tau transgene expression can precipitate neuronal dysfunction and death. These initial studies are encouraging that the zebrafish holds considerable promise as a model in which to study Tauopathies. Further studies are necessary to clarify the phenotypes of transgenic lines and to develop assays and models suitable for unbiased high-throughput screening approaches. This article is part of a Special Issue entitled Zebrafish Models of Neurological Diseases.

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## 1. Introduction

The microtubule-associated protein Tau (MAP- $\tau$ , 'Tau') undergoes biochemical alterations, cellular redistribution, and deposition as insoluble intraneuronal fibrils (Fig. 1), in a variety of neurodegenerative conditions that are collectively termed 'Tauopathies'. Together, these diseases, which include Alzheimer's disease, progressive supranuclear palsy and other conditions (Table 1), are an important cause of morbidity and mortality, with diverse clinical manifestations. No currently available treatments improve the prognosis of any of these relentlessly progressive diseases. Consequently, investigations aimed at determining the underlying pathophysiology of Tauopathies, and isolating novel therapeutic agents that prevent disease progression, are of great importance. In this review, we consider recent developments concerning the possibility that a zebrafish Tauopathy model might be useful for therapeutic target and drug discovery *in vivo*. After

briefly reviewing current knowledge and murine models of Tauopathy, we discuss the possible advantages of a zebrafish model and whether a truly representative model encompassing key biochemical events underlying Tauopathy can be recapitulated in the zebrafish central nervous system. Finally, we review recent publications demonstrating initial proof of concept that Tauopathy zebrafish models recapitulate core features of the human disorders.

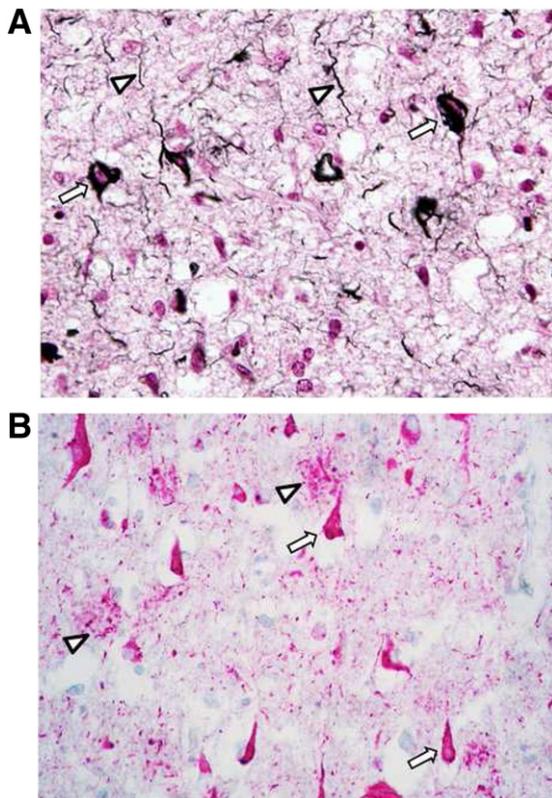
## 2. The microtubule-associated protein Tau

Neurons rely on fast axonal transport to shuttle organelles and macromolecules over long distances, allowing their physiological distribution and turnover within axons and dendrites. Microtubules, which provide the tracks along which molecular motors rapidly transport these diverse cargos, are composed of polymerized tubulin monomers. Assembly of tubulin into microtubules is promoted by microtubule-associated proteins, the first of which to be identified was termed 'Tau' ( $\tau$  was used to denote a factor essential for tubule formation) [1,2]. Tau is expressed widely in neurons, where it is enriched in the axonal compartment [3]. The microtubule-binding domain of Tau localizes to the C-terminal half of the protein [4,5] (Fig. 2). The N-terminal, or projection domain, contains a proline-rich

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**Fig. 1.** Neurofibrillary tangles in Alzheimer's disease. **A:** Neurofibrillary tangles in Alzheimer's disease prefrontal cortex are demonstrated using the Gallyas silver method [127]. NFTs are seen as numerous argyrophilic (black) fibrillar intraneuronal inclusions (arrows). Neuropil threads, axonal abnormalities also caused by accumulations of fibrillar Tau, are also seen (arrowheads). **B:** Neurofibrillary tangles in Alzheimer's disease hippocampus are demonstrated by immunohistochemistry, using an antibody (AT8) that detects phospho-S202/T205-Tau. NFTs are seen as abundant immunoreactive (red) intraneuronal inclusions (arrows). Accumulations of phospho-Tau are also apparent in abnormal axonal terminals surrounding amyloid plaques (arrowheads).

region and multiple potential serine–threonine phosphorylation sites, and is thought to be involved in interactions with other cellular components.

### 2.1. The *MAPT* gene

Tau is encoded by the *MAPT* gene, which is located on chromosome 17 and contains 16 exons. Alternative splicing of the primary transcript leads to a family of mRNAs, encoding different protein

isoforms. In adult human brain, six isoforms are expressed, produced by alternative splicing of exons 2, 3 and 10 (exons 4A, 6 and 8 are not expressed in the CNS). Tau isoforms in the CNS contain either three or four copies of a tandem repeat containing tubulin-binding sequences, referred to as 3R- and 4R-Tau respectively (Fig. 2). Exon 10 encodes the second microtubule-binding repeat, such that inclusion of exon 10 in the mRNA results in translation of 4R-Tau, whereas exclusion of exon 10 results in a transcript encoding 3R-Tau [6,7]. Optional inclusion of exon 2, or exons 2 and 3, gives rise to N-terminal insertions of 29 ('1N') or 58 ('2N') amino acids respectively [8]. The six resulting CNS isoforms are shown in Fig. 2. Additional isoforms generated by incorporation of exon 4A ('large Tau') are expressed in the peripheral nervous system [9].

### 2.2. Functions of Tau

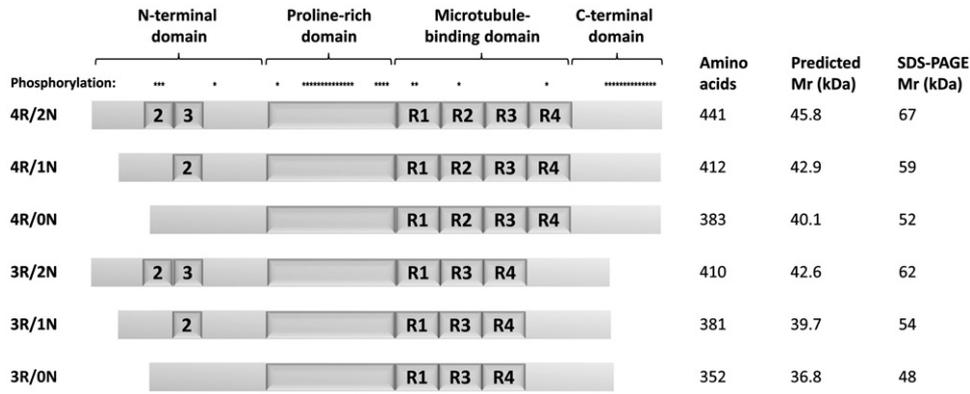
Association of Tau with tubulin promotes microtubule formation [1,2], and association of Tau with microtubules is thought to enhance their stability [10]. 4R-Tau binds to microtubules with greater avidity than 3R-Tau isoforms [4,11,12] (this is attributable to sequences in the first inter-repeat segment that are unique to 4R proteins, rather than to the additional copy of the microtubule-binding tandem repeat [13]), suggesting that expression of 4R-Tau may favor microtubule formation and stability. In healthy adult brain tissue, the 4R-/3R-Tau ratio is approximately 1, and disruption of this balance in the human brain may have serious consequences (see below) [14]. However, there are situations in which the 4R-/3R-Tau ratio may be altered physiologically. For example, during embryogenesis, Tau is exclusively expressed in the brain as the 3R/0N isoform [8]. It has been argued that this might promote plasticity of neuronal processes during development by reducing microtubule stability. Recent work has suggested that Tau interacts with motor proteins involved in axonal transport, provoking different functional consequences for distinct types of motor molecules [15]. Kinesin, which transports cargo towards the distal end of the axon, tended to detach from the microtubule on encountering Tau, whereas the proximally-directed motor Dynein showed more directional reversal than detachment. These effects were apparent in the presence of 3R/0N Tau, and suggest that association of Tau with microtubules may modulate axonal transport in addition to promoting microtubule stability.

### 2.3. Tau phosphorylation

Tau undergoes post-translational changes, including physiological serine–threonine phosphorylation [16] and O-glycosylation [17], in addition to tyrosine phosphorylation [18], SUMOylation [19] and pathological nitration [20]. Of these changes, phosphorylation has

**Table 1**  
Neurodegenerative diseases associated with prominent Tau pathology.

Disease	Typical clinical presentations	Typical Tau pathology	Tau filaments	Tau species	Etiology
Alzheimer's disease (AD)	Memory disorder, dysphasia, frontal lobe cognitive–behavioral disorder, dementia	Flame-shaped neurofibrillary tangles	Paired helical filaments, straight filaments [123]	4R- and 3R-Tau	Mostly unknown, few cases caused by mutations in <i>APP</i> , <i>PS1</i> or <i>PS2</i> genes
Progressive supranuclear palsy (PSP)	Falls, rigidity, oculomotor disorder, cognitive–executive disorder	Globose neurofibrillary tangles, tufted astrocytes	Straight filaments [124]	Predominantly 4R-Tau	Unknown, linked to H1 haplotype at <i>MAPT</i> locus
Corticobasal degeneration (CBD)	Dystonia, myoclonus, apraxia, cortical sensory loss, dementia	Ballooned neurons, pre-tangles, astrocytic plaques	Twisted ribbon filaments [125]	Predominantly 4R-Tau	Unknown, linked to H1 haplotype at <i>MAPT</i> locus
Pick's disease (PiD)	Frontal lobe cognitive–behavioral disorder, dementia	Pick bodies	Helical filaments; straight filaments [126]	Predominantly 3R-Tau	Unknown
Fronto-temporal dementia and parkinsonism linked to chromosome 17 (FTDP17)	Variable Parkinsonian motor disorder and/or frontal lobe dementia	Variable	Variable	Variable (see text)	Mutations in <i>MAPT</i> gene encoding Tau



**Fig. 2.** Isoforms of the microtubule-associated protein Tau. The schematic depicts the six Tau isoforms expressed in the adult human brain, labeled to the left of each protein. Positions of major protein domains are shown above the longest isoform. The N-terminal insertions encoded by exons 2 and 3, and the four tandem repeats (R1–4) in the microtubule-binding domains are indicated. Sites of known phosphorylation are shown above the longest isoform. The table to the right of the figure shows the number of amino acids, predicted molecular mass and electrophoretic mobility of each protein isoform.

been most extensively studied, since pathological hyperphosphorylation is associated with Tauopathy [21]. Approximately 45 phosphorylation sites have been identified, which cluster in the proline rich C-terminal domains of the protein [22]. Phosphorylation of Tau, in particular at S262 and S356 within the microtubule-binding domain, promotes its detachment from microtubules, suggesting a potential mechanism in regulating microtubule stability and axonal transport [23,24]. In addition to microtubule-binding affinity, phosphorylation may have other effects, such as modulating binding to the chaperone Pin1 [25] and possibly in regulating putative signal transduction functions of Tau through phosphorylation of SH3 domains [26].

Hyperphosphorylation of Tau is a characteristic feature in Tauopathy specimens. No single phosphorylation site is specific for Tauopathy. Although some phospho-epitopes may be more readily detected in pathological specimens compared with non-Tauopathy material, most of the known phosphorylation sites have been demonstrated in normal biopsy material [16]. Hyperphosphorylation is a quantitative rather than qualitative phenomenon, defined by the degree to which Tau is phosphorylated at multiple sites. Phosphorylation is a dynamic process and Tau is rapidly de-phosphorylated *post mortem*, accounting for the lack of detectable phospho-epitopes in control autopsy specimens. It is unclear whether a functional abnormality of phosphatase activity is present in Tauopathy samples, or whether the failure of de-phosphorylation in Tauopathy autopsy material is attributable to changes in the properties of the hyperphosphorylated molecule as a phosphatase substrate. During normal development, Tau is present in a highly phosphorylated state [27,28], suggesting that the balance of Tau phosphorylation and de-phosphorylation can change physiologically, without necessarily provoking neurodegeneration.

#### 2.4. Tau kinases

Several cellular kinases are able to phosphorylate Tau *in vitro* or in cell culture (Table 2; reviewed in [22]). Many of these phosphorylate multiple residues of Tau, and individual residues can be phosphorylated by multiple different kinases, suggesting that Tau is a promiscuous kinase substrate. Consequently, it is difficult to be certain which kinase(s) are responsible for physiological or pathological phosphorylation of Tau *in vivo*. Kinases that have emerged as strong *in vivo* candidates include glycogen synthase kinase-3 $\beta$  (GSK3 $\beta$ ) [29–33] and cyclin-dependent protein kinase-5 (cdk5) [34,35]; both are expressed abundantly in neurons and associate with microtubules (cdk5 is anchored to microtubules by Tau [36]). However, it is likely that more than one kinase participates in the events leading to hyperphosphorylation, since no single kinase

phosphorylates all of the residues found in Tauopathy specimens. Furthermore, there are examples *in vitro* of cooperation between kinases; for example, cdk5-mediated phosphorylation of Tau promotes further phosphorylation by GSK3 $\beta$  [37,38]. Consequently, it is thought that the transition from normal to hyperphosphorylated Tau may be a hierarchical or sequential process dependent on phosphorylation by multiple different kinases. Several different protein phosphatases dephosphorylate Tau *in vitro*, including PP1, PP2A, PP2B and PP2C. Their specificities are overlapping and their roles *in vivo* are incompletely understood. PP2A has emerged as a strong candidate, since it appears to be the major brain-derived phosphatase activity directed towards Tau in the rat brain [39] and is localized to microtubules (both through an association with Tau and also directly, which may regulate its activity *in vitro* [40]).

#### 2.5. Tau deposits in sporadic neurodegenerative diseases

Tauopathies are characterized pathologically by the presence of fibrillar deposits of hyperphosphorylated Tau. The histological appearance and distribution of Tau deposits differs between diseases, as does the ultrastructural appearance of Tau filaments that compose neurofibrillary tangles, Pick bodies and other inclusions (Table 1). Formation of neurofibrillary tangles (NFTs) likely occurs through oligomeric intermediates, and there is some evidence that these are the primary pathogenic species, rather than mature NFTs (see below).

The predominant Tau isoforms deposited in neurons differ between diseases. In Alzheimer's disease, SDS-PAGE analysis showed three major

**Table 2**  
Phylogenetic conservation of kinases implicated in tau phosphorylation.

Tau Kinase	Protein accession numbers		% identity	% consensus
	Human	Zebrafish		
Glycogen synthase kinase 3 $\beta$ (GSK3 $\beta$ )	NP_001139628	NP_571456	94.5	97.4
Cyclin-dependent kinase 5 (cdk5)	AAP35326	AAH85381	96.2	98.6
Mitogen activated protein kinase 1 (MAPK1, ERK1)	NP_620407	BAB11813	90.8	93.5
Microtubule Affinity Regulating Kinase 1 (MARK1)	CAH72462	AAI55560	79.3	85.5
Casein kinase 1 $\alpha$ (CK1 $\alpha$ )	NP_001883	NP_694483	99.3	99.3

Tau immunoreactive bands of 68, 64 and 60 kDa present in detergent-extracted material containing paired helical filaments [41]. After dephosphorylation of the same material, all six Tau isoforms were resolved by electrophoresis, showing that hyperphosphorylation of Tau was responsible for the alteration in its electrophoretic mobility, and that AD filaments are composed of all six Tau isoforms. Similar analyses of specimens derived from other types of Tauopathy have revealed different results. In progressive supranuclear palsy and cortico-basal degeneration, two major Tau bands of 68- and 64 kDa are derived from hyperphosphorylation of 4R-Tau species [42,43]. Conversely, material derived from Pick's disease brain shows two bands of 64- and 60-kDa, dephosphorylation of which reveals the presence of 3R-Tau species [43,44]. Interestingly, genetic data shows association between an extended haplotype of markers across the *MAPT* locus, and PSP/CBD [45]; recent data shows that this haplotype favors the splicing of exon 10<sup>+</sup> transcripts encoding 4R-Tau [46], suggesting that genetically-determined relative over-production of 4R-Tau might be one factor in the pathogenesis of these sporadic diseases.

### 2.6. Hereditary fronto-temporal dementia and Parkinsonism linked to chromosome 17

Definitive evidence that the development of Tau pathology may be mechanistically important in common sporadic Tauopathies comes from the study of rare families, in which mutations within the *MAPT* gene are sufficient to cause a neurodegenerative disease with prominent Tauopathy [14,47]. FTDP17 is inherited as an autosomal dominant trait and can manifest as a Parkinsonian movement disorder or as a cognitive-behavioral disorder, resembling other forms of fronto-temporal dementia (reviewed in [48]). To date, 39 different pathogenic *MAPT* mutations have been identified in FTDP17 families. Mutations clustered around the 5' boundary of intron 10 alter the regulation of splicing, most frequently resulting in relative over-production of exon 10<sup>+</sup> transcripts and deposition of hyperphosphorylated 4R-Tau isoforms. Missense mutations within exon 10 also provoke a 4R-Tauopathy, by changing the biophysical properties of 4R-Tau without altering the ratio of splice isoforms; these mutations also result in 4R-Tau deposition. Finally, mutations outside exon 10 are expressed in both 3R- and 4R-Tau, but may provoke selective deposition of 3R- or 4R-, or both isoforms of Tau. A variety of mechanisms has been proposed to account for the pathogenicity of *MAPT* missense mutants, including promotion of Tau fibril formation, impairment of microtubule-binding and introduction or removal of key phosphorylation sites [49]. Many of the mutants show multiple functional abnormalities, such as alterations in 4R-/3R-splice ratio and reduced propensity to promote the stabilization of microtubules.

Although FTDP17 is an uncommon disease, the discovery of pathogenic *MAPT* mutations has provided important confirmation that primary abnormalities of Tau, including alterations in the relative abundance of 3R- and 4R-isoforms, can result in neurodegeneration and Tauopathy. Given the close similarity between FTDP17 and sporadic Tauopathies in some cases, it is reasonable to conclude that abnormalities of cellular Tau metabolism may be central to neurodegeneration in common sporadic Tauopathies. Consequently, study of model systems in which Tauopathy is induced by molecular manipulations that mimic the genetic mechanisms of FTDP17 might yield important insights into pathophysiology and identify potential treatment targets for common sporadic Tauopathies.

### 3. Transgenic mouse models of Tauopathy

A variety of different transgenic lines, over-expressing wild-type or FTDP17 mutant human Tau, have been described (reviewed in [50,51]). Several important themes that have emerged from studies of these animals are briefly considered here.

First, dissociation between NFT formation and neuronal dysfunction or death was demonstrated in some of these models, suggesting that Tau hyper-phosphorylation, relocalization or oligomerization may be more critical for the emergence of phenotypic abnormalities in these models than formation of NFTs. Abnormalities of hippocampal synaptic protein expression and physiology preceded the formation of NFTs in mice expressing P301S mutant Tau [52]. Studies using a different model in which P301L Tau was expressed under an inducible promoter showed that suppression of transgene expression after the onset of phenotypic abnormalities prevented neuronal loss and behavioral abnormalities but did not affect neurofibrillary tangle formation [53], and there was regional dissociation between NFT formation and cell loss [54]. In addition, immunization of Tau/A $\beta$  transgenic mice reduced soluble Tau in the brain but did not affect NFT formation – the animals showed phenotypic improvement, suggesting that neurobehavioral abnormalities were attributable to a soluble Tau species rather than NFT [55].

Second, Tau transgenic mice have provided a powerful means to test the interactions between different biochemical pathways implicated in neurodegeneration. For example, evidence of an *in vivo* interaction between Tau and A $\beta$  in the pathogenesis of Alzheimer's disease was demonstrated by intracerebral inoculation of the Alzheimer's disease-associated amyloid peptide A $\beta$ 1–42, which enhanced the formation of NFTs in a mouse P301L Tau-expressing model [56]. Furthermore, P301L Tau/APP double transgenic animals showed exacerbation of NFT pathology in the presence of the APP transgene, whereas A $\beta$  plaque formation was unaffected by the over-expression of mutant Tau, suggesting that  $\beta$ -amyloid formation or another function of APP might be upstream of Tauopathy in this model [57].

Finally, murine models have provided powerful means to test hypotheses concerning experimental treatment approaches, and biochemical interventions aimed at understanding disease pathogenesis *in vivo* (reviewed in [58]). For example, administration of Li<sup>+</sup>, an inhibitor of GSK3, reduced hyperphosphorylation of Tau *in vivo* and ameliorated some phenotypic abnormalities [31], and another kinase inhibitor targeting cdk5, GSK3 and MAPK1 delayed the onset of a motor deficit in Tau transgenic animals [59]. Other studies have deployed pharmacological interventions in order to clarify the potential therapeutic utility of interventions directed at restoring loss of Tau microtubule-stabilizing function [60], enhancing clearance of pathological Tau species from neurons by inhibiting refolding functions of the HSP90 complex to promote Tau degradation [61], and curtailing a potentially pathogenic neuroinflammatory response by targeting microglial activation using immunosuppressive drugs [52].

Together, these observations show that experimental genetic manipulations, which mimic the mechanisms underlying FTDP17 in humans, provoke phenotypic changes relevant to human Tauopathy in transgenic animals. These models have been valuable for hypothesis-driven experiments aiming to elucidate the pathogenesis of Tauopathy and for testing putative therapeutic approaches.

### 4. Why would a zebrafish Tauopathy model be useful?

The zebrafish is a small freshwater fish that has been used extensively in laboratory studies of vertebrate development. Fish embryos develop externally allowing direct observation of embryogenesis. Many morphological and molecular parallels are shared between fish and other vertebrates allowing insights gained in the zebrafish model to be applied to other systems. Stemming from the use of zebrafish in developmental studies, an extensive array of experimental methodologies has been developed. Straightforward techniques are available for over-expression [62,63] or targeted knockdown of genes of interest [64], allowing the consequences of altered gene function to be determined readily *in vivo*. The deployment of fluorescent reporters [65] allows direct observation

of morphology or physiology of individual cells *in vivo* [66–68]. The ability of zebrafish to breed regularly and produce sizeable clutches of embryos, and the practicable housing of significant numbers of animals, has allowed development of techniques for large-scale genetic [69–72] and chemical modifier [73–75] screens. These have provided numerous novel molecular insights into development, through unbiased phenotype-driven approaches. More recently, it has been demonstrated that intact zebrafish larvae provide an *in vivo* neurobehavioral platform suitable for screens to find chemical modifiers of vertebrate behavior [76].

It is possible that applying these types of analysis to zebrafish models of neurodegenerative diseases would provide novel molecular insights into disease pathogenesis, and allow identification of potentially therapeutic compounds. In particular, screening studies carried out in the intact vertebrate CNS *in vivo* might uncover drug targets that are not present in cell culture models commonly used for discovery-driven approaches, such as pathogenic mechanisms that are not cell-autonomous, or which are only expressed in end-differentiated neurons. Consequently, there has been significant recent interest in the development of zebrafish models of Tauopathy.

## 5. Is the zebrafish a suitable organism in which to study Tauopathy?

The proposed use of a small aquatic creature to study complex human neurobehavioral diseases naturally raises questions about relevance – will it be possible to generate a truly representative model, with potential to yield important insights into the human disorder? A variety of considerations suggest that the zebrafish may be a good model system in which to study human neurological diseases. With respect to the human CNS, the zebrafish shows conservation of basic brain organization [77] and many key neuroanatomical [78,79] and neurochemical [80,81] pathways of relevance to human disease; the zebrafish CNS contains microglia [68], cells with astrocytic properties [82,83], oligodendrocytes and myelin [84–87], and a blood–brain barrier [88], all of which have been implicated in the pathogenesis of neurological disease. These observations imply that the tissue environment in the zebrafish CNS may present appropriate conditions in which to recapitulate pathological changes representative of the human disorders. In addition, there is a striking degree of phylogenetic conservation of genes implicated in the pathogenesis of neurodegenerative diseases, and a variety of other CNS functions [89–96]. This suggests that it will be possible to provoke neurodegeneration in zebrafish through conserved biochemical pathways that are sufficiently close to those underlying human disorders that findings in the zebrafish model will yield relevant insights into diseases mechanisms. It is noteworthy that the zebrafish genome contains highly conserved orthologues of each of the kinases implicated in Tau phosphorylation (Table 2 and Fig. 3), which may be of particular relevance to Tauopathy in view of the proposed central role of hyperphosphorylation in pathogenesis. Similar considerations apply to other conserved pathways, and it seems likely that findings in zebrafish models will be representative of the mechanisms underlying the pathogenesis of human Tauopathies and will therefore be relevant to their understanding.

### 5.1. Zebrafish paralogues of MAPT

Recent work has identified two paralogues of *MAPT* in zebrafish, annotated *mapta* and *maptb* [97]. Analysis of homology between zebrafish and mammalian MAP-encoding genes, and examination of synteny between zebrafish *mapt* and mammalian *MAPT* loci, strongly suggest that the two zebrafish genes arose from duplication of an ancestral *mapt* locus. A genome duplication event is thought to have taken place during the evolution of teleosts [98]; consequently, there are dual zebrafish paralogues of many mammalian genes [92,99]. It is possible that divergent evolution of the two *mapt* genes has given rise

to functional sub-specialization, suggesting that the zebrafish may be a powerful tool in which to dissect multiple cellular roles of Tau. The two proteins encoded by the zebrafish genes have not yet been detected, but both *mapta* and *maptb* mRNAs were expressed in the developing CNS [97]. A complex pattern of alternative splicing of the *mapta* and *maptb* transcripts suggests that, like human Tau, zebrafish Tau isoforms with different numbers of microtubule-binding repeats are expressed in the CNS, and larger forms of Tau are expressed in the peripheral nervous system. Interestingly, *mapta* gives rise to transcripts encoding 4–6 microtubule-binding repeats, whereas *maptb* is predominantly expressed as a 3-repeat isoform, raising the fascinating possibility that conserved functions of mammalian 3R- and 4R-Tau are distributed between the two zebrafish genes [97].

## 6. Tools for the generation and analysis of zebrafish Tau models

Generation of transgenic Tau zebrafish requires availability of appropriate methods for introducing exogenous DNA into the germline, and resources to direct *MAPT* transgene expression in the desired temporal and spatial pattern.

### 6.1. Techniques for establishment of transgenic lines

Transgenic zebrafish can be generated by micro-injecting linearized plasmid DNA into the cytoplasm of one-cell stage embryos [100], but this gives rise to inefficient genomic integration and significant mosaicism. Two technical advances, meganucleases and transposons, have substantially improved the efficiency by which foreign DNA can be introduced into the zebrafish genome. (i) The meganuclease *I-sce1* is an intron-encoded endonuclease from *Saccharomyces cerevisiae* [101], which cleaves DNA at an 18 bp sequence-specific recognition site not found within the zebrafish genome. In meganuclease-mediated transgenesis, the transgene plasmid is designed so that the expression cassette is flanked by *I-sce1* sites, and is injected into zebrafish embryos with *I-sce1* enzyme. Integration of a low transgene copy number usually occurs at a single site, with substantially enhanced efficiency, and reduced mosaicism, compared with naked DNA injection. This improves the rate of transmission of the transgene from the F0 to the F1 generation [102] and results in simple Mendelian transmission of transgenes in subsequent crosses [103]. (ii) Type 2 transposons are mobile DNA elements encoding a transposase, which mediates excision of the transposon from the genome and its re-insertion at another location. The *Tol2* transposon, found in the genome of medaka [104], has been engineered by deletion of the transposase gene to form a non-autonomous mobile element, which can insert into the genome in the presence of transposase supplied *in trans* [105]. In this approach to transgenesis, the transgene expression cassette is flanked by transposon elements, and injected into zebrafish embryos with mRNA encoding transposase, resulting in highly efficient, usually multiple, single-copy integration events. The technique has been used increasingly since its introduction and subsequent refinement [62], because it is necessary to inject and screen a relatively small number of embryos in order to identify stable transgenic lines.

### 6.2. Promoter resources for generation of neurodegeneration models

Most transgenic zebrafish lines have been constructed by expressing a cDNA under control of a characterized heterologous promoter fragment; *cis*-acting regulatory regions derived from zebrafish genes are usually employed for this purpose, because they may be associated with more reliable transgene expression [106,107]. For the generation of a Tauopathy model, desirable attributes for the promoter would include pan-neuronal activity and sufficient expression to provoke pathology. Three zebrafish promoter elements that drive transgene expression widely in CNS neurons have been described. (i) A neuronal enhancer derived from the *gata2* promoter was expressed at high levels in the

## A. Glycogen synthase kinase 3 $\beta$

NP_001139628 Human GSK3 $\beta$	(1)	MSGRPRRTTSFAESCKPVQPSAFGSMKVS RDKDGSKVTTVVATPGQGPDRPQEVSYDTDKVIGNGSFGVVYQAKLDCSGELVAIKKVLQDKRFKNRELQI	**** * * * *
NP_571456 Zebrafish GSK3 $\beta$	(1)	MSGRPRRTTSFAESCKPVQPSAFGSMKVS RDKDGSKVTTVVATPGQGPDRPQEVSYDTDKVIGNGSFGVVYQAKLDCSGELVAIKKVLQDKRFKNRELQI	
NP_001139628 Human GSK3 $\beta$	(101)	MRKLDHCNIVRLRYFFYSSEKDKDEVYLNLDVDPETVYRVARHYSRAKQTLPMVYVKLYMYQLFRSLAYIHSFGICHRIKPNLLLDPDTAVLKLCD	* **** * ^ *
NP_571456 Zebrafish GSK3 $\beta$	(101)	MRKLDHCNIVRLRYFFYSSEKDKDEVYLNLDVDPETVYRVARHYSRAKQTLPMVYVKLYMYQLFRSLAYIHSFGICHRIKPNLLLDPDTAVLKLCD	
NP_001139628 Human GSK3 $\beta$	(201)	FGSAKQLVRGEPNVSYICSRYYRAPELIFGATDYTSSIDVWSAGCVLAELLGQIFPGDSGVDQVLEIIVKVLGTPREIQIREMNPNYTEFKFPQIKAHP	^ ^ ^ ^ ^
NP_571456 Zebrafish GSK3 $\beta$	(201)	FGSAKQLVRGEPNVSYICSRYYRAPELIFGATDYTSSIDVWSAGCVLAELLGQIFPGDSGVDQVLEIIVKVLGTPREIQIREMNPNYTEFKFPQIKAHP	
NP_001139628 Human GSK3 $\beta$	(301)	WTKVFRPRTPEAIALCSRLLEYTPARLTPLAECAHSFFDELRPVNKLPNGRDTIPALFNFTTQELSSNPPLATILIPPHARLQAAASTPTNATAASDA	
NP_571456 Zebrafish GSK3 $\beta$	(301)	WTKVFRPRTPEAIALCSRLLEYTPARLTPLAECAHSFFDELRPVNKLPNGRDTIPALFNFTTQELSSNPPLATILIPPHARLQAAASTPTNATAASDA	
NP_001139628 Human GSK3 $\beta$	(401)	NTGDRGQTINNAASASASNST	
NP_571456 Zebrafish GSK3 $\beta$	(401)	NSGDRSQTINNAASASASNST	

## B. Cyclin-dependent protein kinase 5

AAP35326 Human cdk5	(1)	MQKYEKLEKIGEGTYGTVFKAKNRETHEIVALKRVRLLDDDDGEGVSSALREICLLKELKHKNIIVRLHDVLSHDKKLLTVFEFCDDQLKKYFDFSCNGDLDP	**** * * * *
AAH85381 Zebrafish cdk5	(1)	MQKYEKLEKIGEGTYGTVFKAKNRETHEIVALKRVRLLDDDDGEGVSSALREICLLKELKHKNIIVRLHDVLSHDKKLLTVFEFCDDQLKKYFDFSCNGDLDP	
AAP35326 Human cdk5	(101)	EIVKSFLLQLLKGLGFCHSRNVLRDLKPNQLINRNGELKLADFGLARAFGIPVRCYSAEVVTLWYRPPDVLFGAKLYSTSIDMWSAGCIFAELANAGR	^ ^ ^ ^ * ^
AAH85381 Zebrafish cdk5	(101)	EIVKSFMYQLLKGAFCHSRNVLRDLKPNQLINRNGELKLADFGLARAFGIPVRCYSAEVVTLWYRPPDVLFGAKLYSTSIDMWSAGCIFAELANAGR	
AAP35326 Human cdk5	(201)	PLFPGNDVDDQLKRIFRLLGTPTEEQWQSMKLPDYKPYMPYPATSLVNVVPKLSTGRDLLQNLKCNPVQRISAEELQHPYFSDFCPP	^
AAH85381 Zebrafish cdk5	(201)	PLFPGNDVDDQLKRIFRLLGTPTEEQWQSMKLPDYKPYMPYPATSLVNVVPKLSTGRDLLQNLKCNPVQRISAEELQHPYFSDFCPP	

### Key:

XXX = identical  
 XXX = conservative  
 XXX = non-similar

\* = ATP binding site  
 ^ = substrate binding site

B = activation loop  
 U = catalytic domain

**Fig. 3.** Phylogenetic conservation of kinases implicated in Tau phosphorylation. The sequence figure shows the striking degree of amino acid conservation between human and zebrafish (A) GSK3 $\beta$  and (B) cdk5. Each panel shows the Human (upper) and zebrafish (lower) protein sequences, aligned using the ClustalW algorithm. Amino acids are shaded to show identical, conserved and non-similar residues. The catalytic domain of each kinase is indicated by underlining, and important functional sites labeled as indicated.

developing CNS [108]. (ii) The zebrafish *huc* gene encodes an RNA binding protein, HuC/D, commonly used as an early neuronal marker [109] – a 2.8 kb fragment of the proximal 5' flanking region was sufficient to drive pan-neuronal expression in embryos [110]. (iii) The *eno2* gene, encoding the neuron-specific  $\gamma$ -enolase isoenzyme, is expressed at low levels until 60–72 h post-fertilization, after which high level expression persists into adulthood. The *eno2* regulatory region is complex, containing an untranslated first exon, and an intronic CpG island that appears important for promoter activity. A 12 kb fragment of the promoter, including the first intron, was active in driving reporter gene expression in neurons, including many populations of relevance to disease, throughout the brain and spinal cord from 36–48 h post-fertilization through adulthood [111], and in the retina and visual pathways [103] (Fig. 4). The optimal promoter for construction of Tauopathy models is unclear, and each of these promoters has been deployed in studies of transgenic Tau zebrafish (see below). It is likely that further development of promoter resources and other approaches to generation of transgenic animals will yield other useful reagents for this application.

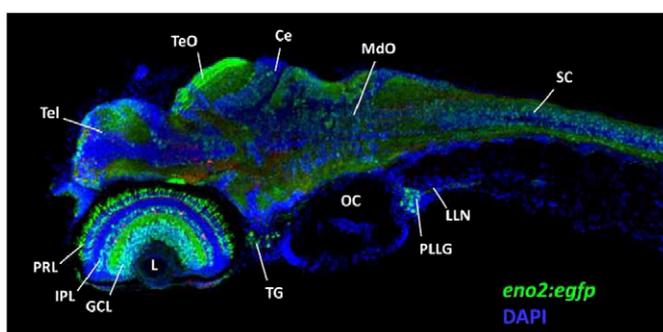
### 6.3. Special considerations for expression of transgenes that provoke neurodegeneration in larvae

In order to fully exploit the potential of the zebrafish for high-throughput screening approaches, it may be necessary to drive Tau expression at sufficiently high levels that disease pathogenesis occurs during larval stages of development, when zebrafish can be accommodated in 96-well plates. However, development of neurodegeneration at this early time point could provide strong selection against establishment of transgenic lines, by compromising repro-

ductive potential or preventing survival to sexual maturity. One possible solution would be deployment of a conditionally-expressing system, for example the binary Gal4–UAS system previously employed in *Drosophila* genetics. This system has been successfully used in zebrafish, with recent modifications attempting to address toxicity issues caused by the viral *trans*-activation domain usually fused to Gal4, and inactivation of the UAS tandem repeats [112,113]. Isolation of useful driver lines for generation of neurodegenerative disease models is ongoing, and the optimal strategy for deployment of this technology is uncertain. Driver lines could be constructed by expressing the Gal4-transactivator fusion protein using small promoter fragments similar to the approach for generating cDNA transgenic animals; this has been used successfully with the *huc* promoter in a larval Tau model [114]. In addition, the highly efficient genomic integration of transgenes using the Tol2 transposon has allowed the deployment of approaches in which random integration of a *gal4*-expressing enhancer trap gives rise to robust stable expression of Gal4 under the endogenous regulatory elements of the gene of insertion [115]. Several such lines have been constructed and are currently being further evaluated.

### 6.4. Assays for fully exploiting transgenic models

The power of screening paradigms might be enhanced by deployment of assays that exploit unique properties of the zebrafish model. First, direct visualization of neuronal populations of interest in the zebrafish brain might allow non-invasive automated imaging to ascertain the morphology, integrity or viability of target cells as an assay end point. For example, the Tauopathies PSP and CBD are associated with severe depletion of dopaminergic neurons causing a



**Fig. 4.** The zebrafish *eno2* promoter. The micrograph shows a single confocal plane through the head region of a Tg(*eno2:egfp*) zebrafish larva at 5 days post-fertilization, illustrating widespread neuronal expression of the *eno2* promoter. Rostral is to left, dorsal up. GFP expression appears green; the section was counterstained using a blue nuclear marker to facilitate identification of anatomical landmarks. Key: Tel, telencephalon; TeO, optic tectum; Ce, cerebellum; MdO, medulla oblongata; SC, spinal cord; L, ocular lens; PRL, photoreceptor layer of retina; IPL, inner plexiform layer of retina; GCL, ganglion cell layer of retina; TG, trigeminal ganglion; OC, otic capsule; PLLG, posterior lateral line ganglion; LLN, axons of lateral line nerve.

Parkinsonian movement disorder that characterizes these conditions [116], and it would be of considerable interest to identify compounds that protect dopaminergic neurons from abnormal forms of Tau. Genetic labeling of dopamine neurons using fluorescent reporters is ongoing, and has proved to be complicated. The promoter regions of dopamine-neuron-specific genes are large and complex, such that manageable fragments of the *th* [117,118] or *slc6a3* [119] promoters have not shown dopaminergic neuron-specific activity *in vivo*. Alternative approaches are being undertaken, including identification of conserved enhancers, using large genomic constructs likely to contain more *cis*-acting elements, or adopting an enhancer trap approach to identify genomic integrants that recapitulate endogenous gene expression patterns. The latter approach has yielded a single transgenic line in which an integration event in the *vmat* gene yielded zebrafish with GFP-expressing catecholaminergic neurons [67]. There is a large literature on the development of promoter resources allowing genetic labeling of other specific neuronal populations using fluorescent reporters, which is outside the scope of the present review.

Second, neuronal dysfunction presumably precedes cell death in Tauopathy, and cells in earlier stages of pathological evolution might present a more tractable therapeutic target than those close to demise. It would be of considerable value to develop assays that measure neuronal dysfunction, and which are sensitive to changes in disease progression earlier in the evolution of pathology. Since genetic and chemical screening against a zebrafish Tauopathy model would be carried out *in vivo*, it might be possible to use automated motor behavioral assays as a surrogate marker of neuronal function. Consequently there is much current interest in establishing reproducible and well-characterized behavioral assays for neurodegenerative phenotypes. A recent report showed that an automated motor behavioral test could be used to identify agents with neuropharmacological properties in a chemical screen [76]. Assays of motor function have been previously deployed in order to detect changes in behavior caused by alterations in neurochemistry and dopamine cell integrity secondary to Parkinson's disease-associated toxins [81,120]. It may be possible to use a similar approach to investigate changes in behavior preceding demonstrable cell loss in a zebrafish Tauopathy model, in order to develop an assay to identify chemical modifiers of early pathological progression.

## 7. Current transgenic zebrafish models of Tauopathy

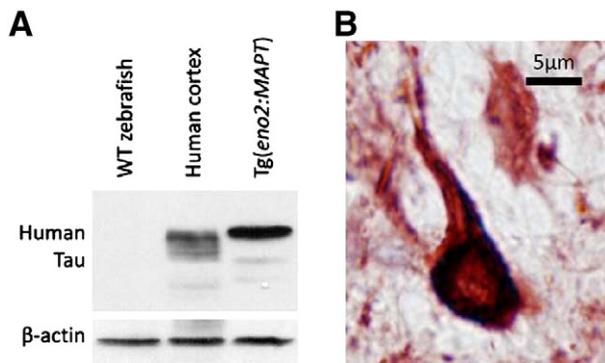
Three recent publications have described the first proof-of-principle experiments showing that expression of human *MAPT* transgenes can provoke relevant phenotypes in zebrafish.

In the first publication, a 4R-Tau-GFP fusion protein was transiently over-expressed in zebrafish larvae, using a *gata2* promoter

element [121]. In cultured cells, the fusion protein was phosphorylated and associated with the cytoskeleton, similar to native Tau; *in vitro* studies showed that the fusion protein aggregated, validating its use in constructing an *in vivo* model of NFT formation. Zebrafish embryos microinjected with the *gata2:MAPT-egfp* construct, showed mosaic neuronal expression of the fusion protein. Some neurons showed accumulation of fluorescent fibrillar structures resembling neurofibrillary tangles, in the cell body and proximal axon. The human Tau-GFP fusion protein was shown to be phosphorylated in the zebrafish brain by western blot detection of a phospho-S396/S404 epitope, and expression of glycogen synthase kinase-3 in the zebrafish embryo and adult brain was demonstrated by western blot. This initial study validated the use of a GFP fusion protein to monitor evolution of tangle pathology *in vivo*, and suggested that human Tau is phosphorylated in the larval zebrafish, confirming the prediction that there is sufficient phylogenetic conservation of endogenous zebrafish kinases to modify the human protein. No stable lines were derived in this study.

Subsequently, stable transgenic zebrafish were constructed expressing human 4R/ON Tau [111], the most abundant Tau species deposited in progressive supranuclear palsy [43,44]. The transgene was expressed under transcriptional control of the *eno2* promoter [111]. The full phenotype of these transgenic fish has not yet been reported, since the initial paper focused on characterization of the novel *eno2* promoter. Abundant expression of human 4R/ON Tau was found in the CNS, persisting into adulthood (Fig. 5A). In the adult brain, refractile Tau accumulations resembling neurofibrillary tangles were found within neuronal cell bodies and proximal axons in CNS regions of pathological relevance to PSP, including the optic tectum (Fig. 5B). Stable expression of the Tau transgene into adulthood in this model will allow analyses designed to test whether age-related pathology accumulates in these transgenic lines, similar to the progressive changes seen in the human disease and recapitulated in some murine models, a potentially important step in validating the model system. Furthermore, widespread neuronal expression of the transgene may allow examination of factors involved in cellular vulnerability to Tauopathy.

More recently, exploitation of the Gal4-UAS system allowed generation of a Tauopathy model showing a larval phenotype [114]. This is potentially an important advance, since the development of phenotypic abnormalities at larval stages of development may allow high-throughput screening, as discussed above. Human 4R/2N-Tau, harboring a P301L mutation, was expressed from a novel bidirectional UAS promoter, allowing simultaneous expression of a red fluorescent reporter in Tau-expressing cells [114]. The high levels of mutant P301L Tau expression provoked by the *huc:gal4-vp16* driver induced a transient motor phenotype during embryogenesis, likely caused by peripheral motor axonal developmental abnormalities. At later time



**Fig. 5.** Human Tau expression in Tg(*eno2:MAPT*) zebrafish neurons. **A:** A western blot was made with protein lysate from wild-type zebrafish brain (lane 1), control post-mortem human cortex (lane 2) and Tg(*eno2:MAPT*) brain (lane 3). The blot was probed using an antibody to human Tau (upper panel) followed by an antibody to  $\beta$ -actin (lower panel). Abundant expression of human 4R-Tau is seen in transgenic zebrafish compared with the six isoforms in normal human brain [111]. **B:** Sections of Tg(*eno2:MAPT*) zebrafish brain were labeled using the antibody to human Tau used in the western blot experiment shown in panel A and a histochemical reaction yielding a red product. The micrograph shows a brainstem neuron with dense Tau immunoreactivity in the cell body and proximal axon, resembling neurofibrillary tangles shown in Fig. 1B [111].

points, after recovery of motor function, enhanced cell death was observed in the spinal cord of transgenic animals. Abnormal Tau conformers were detected using an antibody, MC1, which recognizes a discontinuous epitope present in Tauopathy tissue [122], as early as 32 h post-fertilization; by 5 weeks of age, argyrophilic material was detected in the spinal cord, but the animals were otherwise phenotypically unremarkable at this time point. Subsequent loss of transgene expression prevented the examination of later pathological changes. Tau phosphorylation was detected as early as 32 h post-fertilization. Phospho-Tau specific antibodies labeled cells differentially; some phospho-epitopes, such as phospho-T231/S235 and phospho-S396/S404 were detected widely in Tau-expressing neurons, whereas others such as phospho-S422 and phospho-S202/T205 were initially present in only a small subset of Tau-expressing neurons. Between 2 and 7 days post-fertilization, however, expression of all of these epitopes became widespread in Tau-expressing cells, suggesting that Tau had accumulated multiple phosphorylations in a sequential progression of biochemical changes. Tau phosphorylation was reduced by application of novel high-potency GSK3 $\beta$  inhibitors that were designed to target the human enzyme, confirming that extensive homology between human and zebrafish kinases allows small molecules to interact with orthologous targets from either species. This important study was the first detailed phenotypic description of a stable zebrafish Tauopathy model and showed proof of principle that biochemical changes characteristic of human Tauopathy can be recapitulated in larval zebrafish and modulated by chemical inhibitors.

## 8. Conclusions – what have we learned and what next?

Examination of the first publications allows preliminary assessment of the degree to which transgenic zebrafish models of Tauopathy seem representative of human disorders. Evidence so far suggests that processing of over-expressed human Tau in zebrafish CNS neurons results in similar biochemical and pathological changes to those found in human Tauopathies. These changes include: aberrant localization of Tau to the somato-dendritic compartment; phosphorylation, with an ordered (and possibly sequential) acquisition of phospho-epitopes; adoption of abnormal conformations associated with Tauopathy; and deposition as argyrophilic material. The availability of zebrafish lines that express Tau in the brain through adulthood will allow more detailed biochemical studies of Tau in the zebrafish CNS to characterize

solubility, fibril morphology and further clarify phosphorylation events. However, these data are encouraging that relevant pathways are sufficiently phylogenetically conserved between human and zebrafish to allow recapitulation of cellular processing events relating to Tau in the zebrafish model.

The pathophysiological consequences of human Tau transgene expression in zebrafish CNS neurons are less clear. This is partly because a detailed phenotype has only been reported for a single transgenic Tau zebrafish line [114]. The motor axonal developmental abnormality, motor phenotype and cell death reported in this model have an uncertain relationship to the neuronal dysfunction and neurodegeneration that characterizes human Tauopathy, on account of the onset and subsequent resolution of abnormalities during early development, and the atypical anatomical distribution in the spinal cord and motor neurons. One recurrent finding from the murine transgenic disease model literature is that phenotypes exhibited by cDNA transgenic animals are critically dependent on the promoter elements used to drive transgene expression. The temporal and spatial expression pattern of the *huC* promoter used in this study directed the atypical pattern of phenotypic abnormalities. However, this may be unimportant for the purposes of drug and target discovery relating to cellular mechanisms of Tau toxicity, provided that the molecular mechanisms underlying neuronal dysfunction and death are conserved between the model and the disorder. Further work, including analysis of other zebrafish Tau lines constructed using different promoter elements, will be necessary to clarify this point.

While detailed phenotypic analysis of the first cDNA transgenic Tau models is in progress, it is interesting to consider future directions in the generation of models and approaches for analysis. Use of models based on over-expression of cDNA encoding human Tau would limit discovery of novel molecular interventions to those targeting Tau modifications, Tau deposition or other abnormal effects exerted by Tau on neuronal function. However, there may be upstream targets related to transcription and splicing that would not be represented in cDNA models. For example, changes in the cellular 3R-/4R-Tau ratio, caused by alteration in the regulation of exon 10 splicing, underlie one form of FTDP17 and may contribute to sporadic Tauopathies, where associated haplotypes may alter 3R-/4R-Tau ratios [46]. It is not clear whether the human *MAPT* locus would be appropriately regulated in transgenic Tau zebrafish, allowing expression of physiological levels and patterns of alternatively spliced products. Transgenic zebrafish lines harboring the entire *MAPT* gene would therefore be of considerable interest. Furthermore, since zebrafish evolution may have resulted in the functions of 3R- and 4R-Tau isoforms becoming distributed between two different genes, an indication of whether alterations in the ratio of these forms can be pathogenic in zebrafish might be accomplished by morpholino knockdown experiments.

Initial evidence shows that the zebrafish will most likely be a predictive platform for the discovery and assessment of Tau kinase inhibitors, although arguably the greatest potential strength of the model might be in discovering new therapeutic targets. In order to gain novel insights from screening approaches, end points that are independent of presumptions about underlying mechanisms, such as neurobehavioral analyses or cell death assays, might be deployed. Furthermore, experimental approaches that can be uniquely applied in zebrafish larvae owing to their optical transparency, for example physiological imaging modalities relating to cellular calcium levels, have the potential to yield important insights from hypothesis-driven experiments that cannot be carried out using other available models.

In conclusion, the first reports show that construction of transgenic zebrafish expressing human Tau in CNS neurons is feasible and suggest that the resulting models will be relevant and useful. Consequently, there is cause for cautious optimism that novel zebrafish Tauopathy models may provide important contributions to ongoing efforts to address these common and devastating diseases.

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