A zebrafish model of CLN2 disease is deficient in tripeptidyl peptidase 1 and displays progressive neurodegeneration accompanied by a reduction in proliferation

Fahad Mahmood,1 Sonia Fu,2 Jennifer Cooke,1 Stephen W. Wilson,2 Jonathan D. Cooper3 and Claire Russell1

1 Department of Comparative Biomedical Sciences, Royal Veterinary College, London, NW1 0TU, UK
2 University College London, Department of Cell and Developmental Biology, London WC1E 6BT, UK
3 Paediatric Storage Disorders Laboratory, Department of Neuroscience, Centre for the Cellular Basis of Behaviour, King’s Health Partners Centre for Neurodegeneration, James Black Centre, Institute of Psychiatry, King’s College London, London SE5 9NU, UK

Correspondence to: Dr Claire Russell, Royal Veterinary College, Royal College Street, London, NW1 0TU, UK
E-mail: crussell@rvc.ac.uk

Tripeptidyl peptidase 1 (TPP1) deficiency causes CLN2 disease, late infantile (or classic late infantile neuronal ceroid lipofuscinosis), a paediatric neurodegenerative disease of autosomal recessive inheritance. Patients suffer from blindness, ataxia, epilepsy and cognitive defects, with MRI indicating widespread brain atrophy, and profound neuron loss is evident within the retina and brain. Currently there are no effective therapies for this disease, which causes premature death in adolescence. Zebrafish have been successfully used to model a range of neurological and behavioural abnormalities. The aim of this study was to characterize the pathological and functional consequences of Tpp1 deficiency in zebrafish and to correlate these with human CLN2 disease, thereby providing a platform for drug discovery. Our data show that homozygous tpp1sa0011 mutants (tpp1sa0011/C0/C0) zebrafish display a severe, progressive, early onset neurodegenerative phenotype, characterized by a significantly small retina, a small head and curved body. The mutant zebrafish have significantly reduced median survival with death occurring 5 days post-fertilization. As in human patients with CLN2 disease, mutant zebrafish display storage of subunit c of mitochondrial ATP-synthase, hypertrophic lysosomes as well as localized apoptotic cell death in the retina, optic tectum and cerebellum. Further neuropathological phenotypes of these mutants provide novel insights into mechanisms of pathogenesis in CLN2 disease. Secondary neurogenesis in the retina, optic tectum and cerebellum is impaired and axon tracts within the spinal cord, optic nerve and the posterior commissure are disorganized, with the optic nerve failing to reach its target. This severe neurodegenerative phenotype eventually results in functional motor impairment, but this is preceded by a phase of hyperactivity that is consistent with seizures. Importantly, both of these locomotion phenotypes can be assayed in an automated manner suitable for high-throughput studies. Our study provides proof-of-principle that tpp1sa0011/C0/C0 mutants can utilize the advantages of zebrafish for understanding pathogenesis and drug discovery in CLN2 disease and other epilepsies.

Keywords: tripeptidyl peptidase 1; TPP1; CLN2 disease; late infantile neuronal ceroid lipofuscinosis; lysosomal storage disorder; zebrafish; model

Abbreviation: CLN = ceroid lipofuscinosis neuronal; SCMAS = subunit c of mitochondrial ATP synthase; TUNEL = terminal deoxynucleotidyl transferase mediated dUTP nick end labelling

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Introduction

The neuronal ceroid lipofuscinoses are a group of mostly autosomal recessive inherited lysosomal storage disorders with an estimated prevalence of ~1 in 100,000 in the UK (www.orpha.net/orphacom/cahiers/docs/GB/Prevalence_of_rare_diseases_by_alphabetical_list.pdf). These progressive neurodegenerative diseases are the most common cause of childhood dementia in the UK (Verity et al., 2010) and are traditionally classified according to age of onset: congenital, infantile, late infantile, juvenile and adult onset. Genes for nearly all forms of neuronal ceroid lipofuscinosis have been identified and they are used in the new nomenclature system. For example, when the ceroid-lipofuscinosis, neuronal 2 (CLN2) gene is mutated the most common disease onset is late infantile, hence the disease is called CLN2 disease, late infantile (previously called late infantile neuronal ceroid lipofuscinosis). The CLN genes predominantly encode lysosomal hydrolases, transmembrane proteins or lysosomal transmembrane proteins, with at least one gene remaining to be identified (Siintola et al., 2007; Mole et al., 2011).

The clinical signs of CLN2 disease, late infantile manifest between years 2 and 4 postnatally (Goebel and Wisniewski, 2004). Presenting symptoms are usually seizures accompanied by developmental regression leading eventually to psychomotor retardation, ataxia and spasticity (Santavuori, 1988; Williams et al., 2006). Visual failure is also apparent leading to blindness towards latter stages of the disease (Santavuori, 1988). MRI scans invariably show widespread cortical and cerebellar atrophy with enlarged sulci, as well as gliosis and demyelination of white matter affecting the internal capsule and optic radiation (Boustany, 1992; Autti et al., 1997a, b; Seitz et al., 1998). Furthermore, macular degeneration and bilateral optic nerve atrophy are prominent on ophthalmological examination (Traboulsi et al., 1987). The majority of patients become refractory to anti-epileptic drugs and there are no effective therapies for this condition, which causes death in the early teens.

Mutations in the CLN2 gene at location 11p15 affects the function of the lysosomal serine protease tripeptidyl peptide 1 (encoded by the TPP1 gene) (EC 3.4.14.9) and these mutations are responsible for CLN2 disease (Sleat et al., 1997; Rawlings and Barrett, 1999; Vines and Warburton, 1999). TPP1 is an N-terminal exopeptidase, with limited endopeptidase activity, of the sedolisin family that functions by utilizing a Glu272, Asp276, Ser475 catalytic triad (Junaid and Pullarkat, 2001; Walus et al., 2005). It is synthesized as a 46-48kDa mature peptide from a 66-68kDa precursor (Ezaki et al., 1999; Lin et al., 2001). Furthermore, TPP1 is part of the mannose 6-phosphate glycoproteome and is thus targeted to the lysosome (Sleat et al., 2008a). So far 98 mutations and 24 polymorphisms have been identified worldwide in the CLN2 disease-causing gene (neuronal ceroid lipofuscinoses mutation resource: www.ucl.ac.uk/ncf). As a result of TPP1 deficiency, cells from patients with CLN2 disease accumulate storage material including subunit c of mitochondrial ATP synthase (SCMAS), which may be a direct substrate of TPP1, although other protein and lipid components also accumulate (Palmer et al., 1992; Ezaki et al., 2000).

Amongst the vertebrates, one mouse model (Sleat et al., 2004) and one canine model (Awano et al., 2006) exist for CLN2 disease. Both models reflect the clinical phenotype of CLN2 disease and provide valuable insights into CLN2 disease pathogenesis. However, both model systems are relatively slow to develop, are inaccessible in the early stages of development and it is costly and impractical to generate large numbers of animals for drug discovery. Therefore assessment of early disease pathogenesis has not been performed and high-throughput drug screening for therapeutics development is expensive. An alternative model organism, that develops externally, is relatively cheap to house and breed and is amenable to high-throughput drug discovery, is the zebrafish, Danio rerio.

Zebrafish have been successfully used to model a range of metabolic, neurological and behavioural abnormalities including lysosomal storage diseases, neurodegeneration, epilepsy and retinal degeneration (Lockwood et al., 2004; Milan et al., 2006; Anichertik et al., 2008; Flinn et al., 2009; Sallinen et al., 2009; Moro et al., 2010). The amenability of zebrafish to large-scale mutagenesis has facilitated the generation of mutants in genes orthologous to those causing human disease. The transparent, externally developing zebrafish embryos display rapid development with highly characteristic phenotypes that can be studied from the earliest stages of development. Furthermore, the ability of adult pairs to lay 100–200 embryos per week coupled with high stock densities enables high-throughput assessment of drug efficacy and toxicity (Goldsmith, 2004; Barros et al., 2008; Rieth et al., 2010). In addition, the zebrafish tpp1 gene (Ensembl I.D. ENSDARG00000042793) is 62% identical to the human orthologue whereas the encoded Tpp1 protein is 67% homologous including the conserved Glu360, Asp272, Ser475 catalytic triad (Wlodawer et al., 2003). According to Ensembl, zebrafish Tpp1 is synthesized from 13 exons as a 557 amino acid pro-peptide, and therefore has a similar exon structure and size as seen in humans. The sequence also indicates that the zebrafish pro-peptide, like its human homologue, is likely to be targeted to the lysosome after removal of a 19 amino acid signal peptide. Given all the aforementioned features, zebrafish provide an excellent novel, and complementary, platform for modelling human TPP1 deficiency.

In the present study we demonstrate that embryonic and larval zebrafish homozygous for a premature stop codon mutation in exon 3 of the tpp1 gene (tpp1sa0011; –/–) recapitulate the pathological and behavioural features of the human condition. tpp1sa0011; –/– mutants show phenotypic resemblance to human CLN2 disease including early onset retinal and cerebellar degeneration, as well as motor defects and reduced survival. Furthermore, the degenerative changes correlate with localized apoptosis in the CNS and are accompanied by ubiquitous accumulation of SCMAS in cells and enlarged lysosomes, as seen in human CLN2 disease. In addition, novel defects in neurodevelopment are described, including reduced proliferation in the CNS and disorganized axon pathways, including the optic nerve, spinal motor nerves and the posterior commissure. Finally, we demonstrate a period of increased locomotion that indicates seizures before impaired swimming ability that progresses to complete loss of locomotion, which can be monitored using a digital video-tracking system. This
zebrafish mutant thus provides not only a faithful model of human CLN2 disease, but also a platform for screening of compounds using potentially high-throughput assays such as locomotion, to identify any potentially therapeutic non-toxic compounds. Such compounds could be used in further preclinical trials in mouse or canine models of CLN2 disease, eventually leading to application in human patients.

**Materials and methods**

**Generation and maintenance of mutant and morphant zebrafish**

Zebrafish were housed in a multi-rack aquarium system at the Royal Veterinary College and kept on a constant 14/10 h light/dark cycle at 27-29°C (Westerfield, 2007). The tpp1^{sa0011} carrier and tpp1^{hu3587} carrier zebrafish were created by N-ethyl-N-nitrosourea mutagenesis in the Tübingen strain and were obtained from the Sanger Institute (Cambridge, UK: www.sanger.ac.uk/Projects/D_rerio/zmp/) as an outcross to wild-type Tupalf long fin. To generate tpp1 morphant embryos, 2 ng morpholino anti-sense oligonucleotides (Bill et al., 2009) against tpp1 messenger RNA were pressure-injected into 1-2 cell stage wild-type TupLF strain zebrafish embryos using a glass capillary injection needle. Morpholino sequences used were: tpp1 ATG morpholino (gcaacccgCATtgctttcgtgttcg; ATG is shown in capitals; Gene Tools). Morpholino buffer [58 mM NaCl; 0.1 mM EDTA; 0.003% N-phenyl-2-thiourea] was added and the reaction mix was incubated at 25°C. This was before further incubating the reaction mix in first strand buffer, 10 mM dTT and RNasin [400 U] at 4°C for 5 min. This was followed by denaturing at 95°C for 50 min. The resulting complementary DNA was precipitated twice with 70% ethanol and suspended in 250 μl double distilled H2O. The same procedure was followed for 72 to 120 h post fertilization embryonic genomic DNA extractions with the exception that each embryo was suspended in only 50 μl of lysis buffer and DNA was precipitated using 60 μl of isopropanol. Genomic DNA was then used for PCR.

**Genotyping the tpp1^{sa0011} and tpp1^{hu3587} alleles**

Genomic DNA was extracted as described (Rehbein and Bogerd, 2007). The caudal fin clips of adult fish were obtained after anesthesia in MS222 (Tricaine) and dissolved in 400 μl of lysis buffer (100 mM Tris-HCl; 200 mM NaCl; 0.2% SDS; 5 mM EDTA; 100 μg/ml proteinase K; double distilled H2O) overnight at 55°C. This was followed by proteinase K inactivation at 80°C for 30 min before quenching at 4°C. DNA was precipitated with 500 μl of isopropanol before centrifugation at 6500 rpm for 40 min. The resulting pellet of DNA was washed twice with 70% ethanol and suspended in 250 μl double distilled H2O. Genomic DNA was then used for PCR.

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**Complementary DNA preparation, cloning and sequencing**

Total RNA extraction of zebrafish embryos was performed as described Westerfield (2007). For complementary DNA synthesis, 1 ng to 5 μg of total RNA was incubated with 250 ng of random primers and 0.8 mM (each) dNTPs at 65°C for 5 min. This was before further incubating the reaction mix in first strand buffer, 10 mM dTT and RNasin [400 U] at 25°C for 2 min. Finally 200 U of Superscript II Reverse Transcriptase (Invitrogen) was added and the reaction mix was incubated at 25°C for 10 min before 42°C for 50 min. The resulting complementary DNA was used directly in a standard PCR reaction in 50 μl using GoTaq® (Promega). Primers used were: tpp1 AF1 gaggtgctagcaggaaggag (forward primer in the 5'UTR); tpp1 AR2 caaacggctcaagaaatgtc (reverse primer in exon 3); tpp1 AR1atatgacctggaatc (reverse primer in exon 6). Ten microlitres of the PCR product was separated by electrophoresis using VisiGene™ (Stratagene) for good band separation and visualized on a Bio-Rad UV Transilluminator. PCR products were cloned directly into pCRII-TOPO vector (Invitrogen) and transformed into chemically competent TOP10 Escherichia coli (Invitrogen) as per the manual. White colonies were cultured and their plasmids mini-prepped (Qiagen QIA Spin Miniprep Kit). Plasmid DNA was quantified using a NanoDrop™ ND1000 and sequenced using standard primers at the Wolfson Institute of Biomedical Research (UCL).

Sequences were aligned to tpp1 genomic and transcript sequences using CLC Free Workbench software.

**Western blotting**

Western blotting was performed as previously described (Link et al., 2006; Westerfield, 2007). Briefly, 48 h post fertilization embryos were dechorionated before yolk sac removal by vigorous pipetting in cold Ringers+ solution (116 mM NaCl, mM KCl, 1.8 mM CaCl2, 5 mM HEPES; 0.1 mM EDTA; 0.3 mM phenylmethylsulphonyl fluoride, pH 2.5). The samples were homogenized in 1 x SDS sample buffer (0.125 M Tris-HCl pH 6.8, 20% glycerol, 10% β-mercaptoethanol, 4% SDS) and proteins were denatured at 95°C for 5 min. Protein quantification was performed by NanoDrop spectrophotometry and 50 μg of protein sample as well as 5 μl of Controls™ prestained protein ladder (NEB P7711) markers were loaded onto a 4% polyacrylamide gel (10% SDS). The gel was run at 100 V for 1 h. The proteins were transferred onto a methanol saturated polyvinylidene difluoride membrane using wet-transfer methodology (transfer buffer: 25 mM Tris-base pH 8.5, 0.2 M glycine, 20% methanol). The polyvinylidene difluoride membrane was probed with rabbit anti-TTP1 (human) (gift from Dr J. Tynnelä) (Ezaki et al., 1999) primary antibody (1:900) overnight at 4°C in 5% bovine serum albumin-Tris-buffered saline 0.1% Tween Goat anti-rabbit horseradish peroxidase-conjugated...
secondary antibody (1:3000, Dako, P0048) was subsequently applied for 1.5 h at room temperature. The blot was developed using the ECL immunoblotting protocol (GE Healthcare). Horseradish peroxidase-conjugated anti-GAPDH (1:4000) (gift from Dr M. Campanella) (Abcam, ab9482) was used as a loading control.

**Tpp1 activity assay**

Tpp1 activity was measured as previously described (Sleat et al., 2008b). Zebrafish at 96 h post fertilization were suspended in substrate buffer (150 mM NaCl, 100 mM sodium citrate, 1 g/l Triton™ X-100, pH 4.0) at a ratio of one embryo per 10 µl. The embryos were homogenized using a pestle and the crude protein suspension was quantified using NanoDrop® spectrophotometry. Total protein (g/µg) was added to 0.25 mM Arg-Ala-Phe-ACC (from 25 mM stock in dimethyl sulphoxide; gift from Professor P. Lobel) (Sleat et al., 2008b) in substrate buffer (as above) to a final reaction volume of 50 µl in a single well of a black 96-well plate. The reaction mixture was incubated in the dark for 90 mins before termination by addition of 25 µl of a black 96-well plate. The reaction mixture was incubated in the dark at room temperature. The sections were mounted in TissueTek O.C.T. (VWR, 25608-930) before 30% sucrose impregnation. The embryos were subsequently imaged by scanning laser confocal microscopy (Leica™ SP5) and imaged with the Chromera software. The well chamber temperature ensured even light spread. The well chamber temperature was maintained at 20-22°C overnight. This was taken to represent darting behaviour.

**Whole mount immunofluorescence**

Immunofluorescence staining was performed as previously described by (Westerfield, 2007). 1-phenyl-2-thiourea-treated embryos were digested in 0.25% trypsin for 8 min on ice or 10 µg/ml protease K for 1 h at room temperature following fixation in 4% paraformaldehyde/PBS. The embryos were subsequently washed in PBS + 0.8% Triton™ X-100 and blocked in 10% normal goat serum: 1% dimethyl sulphoxide in PBS + 0.8% Triton™ X-100 blocking solution for 1 h at room temperature. This was followed by incubation with primary antibody at 4°C overnight (anti-acetylated mouse alpha-tubulin (1:1000, Sigma, T6793), rabbit anti-ph3 (1:1000, Upstate, 06-570), mouse anti-HuC/D (1:1000) (Molecular Probes, A21271), rabbit anti-SCMAS (1:1000) (gift from Dr J. Tyynela¨) (Suopanki et al., 2004), or rabbit anti-GFAP (gift from Sam Nona and John Scholes) (Heath and Xavier, 2009)) in blocking solution. Following several washes, the embryos were incubated at 4°C overnight in an appropriate fluorescent secondary antibody [goat anti-rabbit Alexa Fluor® 488, (1:200, Invitrogen, A10034) or goat anti-mouse Alexa Fluor® 488 (1:200, Invitrogen, A11001)]. Fluorescently-labelled embryos were imaged by scanning laser confocal microscopy (Leica™ SP5) and images subsequently manipulated using Adobe Photoshop. All images for the same antibody were acquired and manipulated in exactly the same way.

**Immunofluorescence staining of zebrafish embryo sections**

Tpp1 immunofluorescence staining was performed as described (Westerfield, 2007). Briefly, 1-phenyl-2-thiourea-treated zebrafish embryos were fixed in 4% paraformaldehyde/PBS overnight at 4°C before 30% sucrose impregnation. The embryos were subsequently mounted and frozen in TissueTek O.C.T. (VWR, 25608-930) in liquid nitrogen before being transverse-sectioned into 10 µm slices on a cryostat and collected onto Superfrost™ PLUS slides. Following a 10 min wash in PBS, the sections were permeabilized in 100% methanol for 10 min before three 5 min washes in PBS + 0.1% Tween-20. The sections were blocked for 1 h with 1.5% normal goat serum in PBS + 0.1% Tween-20, before overnight incubation with rabbit anti-TPP1 (1:600) (see above) in blocking solution at 4°C. After a further three washes for 5 min in PBS + 0.1% Tween-20, the sections were incubated in secondary antibody (goat anti-rabbit Alexa488 Fluor® (as above)) in PBS + 0.1% Tween-20 for 1 h in the dark at room temperature. The sections were mounted in 70% glycerol before imaging using the Leica SP5 Confocal.

**TUNEL assay**

Apoptosis was detected by a terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) assay (Gavioli et al., 1992; Williams and Holder, 2000). Dechorionated 1-phenyl-2-thiourea-treated embryos between 48 and 72 h post fertilization were fixed in 4% paraformaldehyde/PBS overnight at 4°C followed by washing in PBS + 0.1% Triton™ X-100 and subjected to proteinase K digestion according to the age of the embryo (48 h post fertilization, 10 ng/µl for 1 h; 72 h post fertilization, 15 ng/µl for 1 h in PBS + 0.1% Triton™ X-100). Subsequently, the embryos were fixed for 20 min in 4% paraformaldehyde and blocked in Equilibration Buffer (Millipore™) in a 1 h overnight incubation at 37°C in TdT reaction mix (Reaction Buffer 2, Chemicon™ and TdT enzyme, Chemicon™, in a ratio of 2:1) and 0.3% Triton™ X-100. Subsequent to several washes in PBS + 0.1% Triton™ X-100 the embryos were blocked in MA Block [150 mM NaCl, 100 mM malic acid, 2% blocking reagent (Boehringer Mannheim), pH7.5] for 2 h at room temperature and then incubated overnight in anti-DIG-alkaline phosphatase Fab fragments (1:3000 in MA Block). The DNA fragments were resolved using the chromogenic substrate NBT (18.75 mg/ml)/BCIP (9.4 mg/ml) (Roche) [20 µl in 1 ml Buffer B3 (0.1 M Tris-HCl, 50 mM MgCl2, 0.1 M NaCl, 0.1% Tween20)]. TUNEL-labelled embryos were imaged using a Leica™ DM IRB inverted microscope. The number of apoptotic cell bodies in TUNEL stained embryos were quantified within the whole eye, the forebrain, mid-hindbrain and spinal cord (somites 14–20) using ImageJ software.

**Locomotion analysis**

Individual 96 h post fertilization tpp1sa0011−/− or wild-type fish were placed in 50 µl of aquarium water within a single well of a 96-well plate, mounted on the stage of a DMI-600 brightfield microscope. One hundred and seventy second-long video recordings of individual fish were obtained with the Leica DFC-350 FX camera (frame rate: 20 frames/s) with an exposure time of 82 ms. A diffuser fitted within the microscope ensured even light spread. The well chamber temperature was kept constant at 28°C and humidified during the recording period using 5% CO2 (200 bar pharmaceutical grade, BOC) delivered at 1/1 min. For locomotion tracking of 72 h post fertilization larvae, individual tpp1sa0011−/− or wild-type fish were placed in 50 µl of aquarium water within a single well of a 96-well plate, mounted on a Nikon SMZ1500 stereomicroscope and recorded for 20 min using a Digital Sight DS-2 Mv camera (30 frames/s) in a room kept at 25°C. Various movement parameters of individual zebrafish were quantified using Ethovision XT (Noldus) software. Movement bouts were defined as the period between which the average velocity of zebrafish exceeded the user-defined Start Velocity of 0.4 mm/s and remained moving until the user defined Stop Velocity of 0.2 mm/s over a predefined averaging interval of five samples. This was taken to represent darting behaviour.
All statistical analysis was performed using GraphPad Prism 5.00 except survival assessment, which was performed using SPSS v7.

Results

Zebrafish tpp1 mutant homozygotes show outward signs of neurodegeneration

Zebrafish carriers of the tpp1<sup>4a0011</sup> and tpp1<sup>hu3587</sup> mutant alleles (tpp1<sup>4a0011+/−</sup> and tpp1<sup>hu3587+/−</sup>, respectively) generated by N-ethyl-N-nitrosourea mutagenesis were obtained and verified by PCR and direct sequencing of genomic DNA from adult fish fin-clips. The tpp1<sup>4a0011</sup> allele has a single T>A base pair change in exon 3 (Fig. 1C and D), resulting in an in-frame, premature stop codon within the tpp1 gene and transcript (Fig. 1B). The tpp1<sup>hu3587</sup> allele has a single T>A base pair change (Fig. 1F and G) that encodes an in-frame stop codon in exon 11 (Fig. 1E). A comparison of the tpp1 transcripts generated from the wild-type, tpp1<sup>4a0011</sup> and tpp1<sup>hu3587</sup> alleles is shown in Fig. 1A.

All tpp1<sup>4a0011+/−</sup> matings (n = 10) gave approximately one-quarter of embryos (28%) with an abnormal phenotype at 72 h post fertilization, indicating autosomal recessive inheritance (Fig. 1I). We named these embryos ‘putative tpp1<sup>4a0011</sup> wild-type’, tpp1<sup>4a0011</sup> (Fig. 1I). We named these embryos ‘putative tpp1<sup>4a0011</sup> wild-type’, tpp1<sup>4a0011</sup> (Fig. 1I) and their normal looking siblings ‘wild-type’. More detailed inspection revealed that the abnormal phenotype could be first observed at 48 h post fertilization, following a period of apparently normal development, after which severe developmental abnormalities appeared, characterized by an increasingly curved body, small eyes and a shrunken head (Fig. 2A and C). Furthermore, by 72 h post fertilization there was no detectable jaw as well as signs of pericardial oedema (Fig. 2B and D). At later stages the swim bladder was not detectable in tpp1<sup>4a0011+/−</sup> embryos (data not shown).

The progressive reduction in eye size is an early and prominent feature that we show to be statistically significantly different in putative tpp1<sup>4a0011+/−</sup> embryos compared with wild-type. The mean retinal area of putative tpp1<sup>4a0011+/−</sup> embryos was significantly different from wild-type siblings (P < 0.001, unpaired t-test) (n = at least four each) (Fig. 2L). By 72 h post fertilization this difference was even greater (P < 0.001, unpaired t-test) as the putative tpp1<sup>4a0011+/−</sup> showed little change since 48 h post fertilization but the wild-type retinal area increased (Fig. 2L) (n = at least four each). Importantly, genotyping confirmed that putative tpp1<sup>4a0011+/−</sup> larvae (n = 13) were homozygous for tpp1<sup>4a0011</sup> (Fig. 1D).

We next examined the phenotype of a second zebrafish tpp1 mutant, by examining offspring from increasing fish heterozygous for the tpp1<sup>hu3587</sup> allele (tpp1<sup>hu3587+/−</sup>). Only 9.4% (n = 6 crosses) (data not shown) of tpp1<sup>hu3587+/−</sup> offspring displayed a similar morphological phenotype to putative tpp1<sup>4a0011+/−</sup> (Fig. 2E and F), which is less than expected for autosomal recessive inheritance and suggests a weaker allele. We raised the tpp1<sup>hu3587+/−</sup> offspring to 3 months old, genotyped them (Fig. 1G) and found that most tpp1<sup>hu3587+/−</sup> zebrafish had survived. Finally, when tpp1<sup>hu3587+/−</sup> and tpp1<sup>4a0011+/−</sup> were inter-crossed, approximately one-quarter of the offspring (Supplementary Fig. 1G) displayed an abnormal phenotype similar to that displayed by tpp1<sup>4a0011+/−</sup> larvae, but their disease progression was more protracted. Morphological abnormalities were first evident in these compound mutant fish at 72 h post fertilization and by 120 h post fertilization larvae had small eyes and brain as well as loss of jaw and swim bladder compared with normal siblings (Fig. 2G and Supplementary Fig. 1A–F). Furthermore, genotyping confirmed that morphologically abnormal larvae were heterozygous for tpp1<sup>4a0011</sup> and tpp1<sup>hu3587</sup> alleles (n = 3) (Supplementary Fig. 1H and I).

To further confirm that the abnormal phenotype seen in tpp1<sup>4a0011</sup> homozygotes is due to mutation in tpp1 and no other genes, we examined if the phenotype was recapitulated in Tpp1 morpholino knock-down embryos. Injecting 2 ng of tpp1 SPL morpholino (aberrantly splicing the tpp1 messenger RNA transcript) or tpp1 ATG morpholino (inhibiting tpp1 messenger RNA translation) into wild-type fish resulted in an abnormal phenotype beginning around 48 h post fertilization including increasingly smaller eyes and head, curvature within the body axis, a reduced jaw (Fig. 2H–K) and no swim bladder (data not shown), similar to that seen in tpp1<sup>4a0011+/−</sup> zebrafish.

Western blotting confirmed the specificity of the TPP1 antibody for the zebrafish Tpp1 protein and a loss of Tpp1 in tpp1 mutants and morphants. A band of 46 kDa, the expected size for mature Tpp1 (Ezaki et al., 1999), was observed in wild-type 48 h post fertilization embryos from a tpp1<sup>4a0011+/−</sup> incross. Near complete absence of Tpp1 protein was observed in abnormal 48 h post fertilization tpp1<sup>4a0011+/−</sup> embryos from the same tpp1<sup>4a0011+/−</sup> incross, and also in wild-type embryos injected with 2 ng of the tpp1 ATG morpholino (Fig. 1J).

To further demonstrate that tpp1 knockdown results in the morphologically abnormal phenotype, tpp1 SPL morpholino injected embryos raised to 24 h post fertilization were assayed for aberrant tpp1 splicing. Reverse transcriptase-PCR using a forward primer against the 5’-UTR region (Tpp1 AF1) and a reverse primer either in exon 3 (Tpp1 AF2) or exon 6 (Tpp1 AF6) gave additional bands in tpp1 SPL morpholino injected fish (Fig. 1H) compared with uninjected controls. The extra bands were sequenced and two novel transcripts were found, one corresponding to the retention of intron 2, which contains a stop codon, and one corresponding to the loss of exon 2 (Fig. 1A). Therefore the tpp1 SPL morpholino causes two abnormal tpp1 transcripts to be produced.

We next measured Tpp1 activity in whole protein derived from abnormal 96 h post fertilization tpp1<sup>4a0011+/−</sup> mutants and wild-type siblings from a tpp1<sup>4a0011+/−</sup> incross using the fluorescent substrate Arg-Ala-Phe-ACC (Sleat et al., 2008b). As any maternally derived Tpp1 protein would be found in the lysosomes, and because zygotically derived mutant Tpp1 would not reach the lysosome as it does not have a signal sequence, we decided to use a crude whole protein extract rather than a lysosomal preparation. Tpp1 activity was significantly reduced (P < 0.0001) in putative tpp1<sup>4a0011+/−</sup> embryos compared with wild-type embryos after 90 min incubation with 6 μg of crude protein extract (Fig. 1K).

Lastly, immunofluorescence staining for Tpp1 was performed in 48 h post fertilization wild-type and tpp1<sup>4a0011+/−</sup> embryos to confirm Tpp1 deficiency in situ. Tpp1 is present throughout the...
Figure 1 Manipulations of *tpp1* cause Tpp1 deficiency. (A) Diagram illustrating the *tpp1* gene transcript in wild-type (WT), *tpp1*^{sao011}^{−/−} mutant and *tpp1* SPL morphants. The horizontal lines indicate untranslated regions whereas coloured blocks indicate exons. *tpp1*^{sao011} and *tpp1*^{h3587} predicted peptide sequences indicate the presence of a premature stop within peptides derived from these transcripts whereas *tpp1* SPL morpholino (MO) causes the retention of intron 2 in some transcripts (MO T1) and excision of exon 2 in other transcripts (MO T2). Genomic DNA extracted from adult fin clips (B and C) or embryos (D) was PCR amplified and sequenced in the region of exon 3 of the *tpp1* gene to identify wild-type (B), *tpp1*{sao011}^{+/−} heterozygous carriers (C) and *tpp1*^{sao011}^{−/−} homozygous mutants (D). The (continued)
wild-type embryo but we focused on the retina as it has the most overly recognizable structures in these sections and is clinically relevant. We detected Tpp1 in all layers of the wild-type retina, particularly in the outer nuclear layer and ganglion cell layer, whereas Tpp1 levels were markedly absent in 48 h post fertilization tpp1sa0011–/– retina (Fig. 3) in correlation with western blot analysis.

Taken together, these data confirm that the homozygous tpp1sa0011–/– phenotype is caused by mutation in tpp1, and not by any other mutations that may be present in the tpp1sa0011–/– carriers. Importantly, the tpp1sa0011–/– phenotype is fully penetrant and consistent with a phenotype expected for a neurodegenerative disease.

**Tpp1 deficient zebrafish die prematurely**

Humans with the late infantile form of CLN2 disease die in or before the second decade of life (Chang et al., 2011). Therefore, we sought to determine when Tpp1 deficient zebrafish died by performing Kaplan-Meier survival analysis. The tpp1sa0011–/– zebrafish started to die from 4 days post fertilization and none were surviving at 7 days post fertilization. All wild-type and tpp1hu3587+/− embryos were alive at 7 days post fertilization at which point the experiment was halted (Fig. 1L). The median survival for tpp1sa0011–/– larvae was calculated as 5 days post fertilization and log-rank (Mantel-Cox) analysis to test the null hypothesis of no difference in survival functions between wild-type and tpp1sa0011–/– larvae gave a P-value < 0.0001. Thus the mutant phenotype can be correlated directly with significantly reduced survival prospects.

**Ubiquitous storage of subunit c of mitochondrial ATP synthase in tpp1sa0011–/– mutants**

Storage material accumulation, in particular stored subunit c of mitochondrial ATP synthase (SCMAS) within lysosomes, is characteristic of CLN2 disease pathology (Palmer et al., 1992, 1997; Westlake et al., 1995). Forty-eight hours post fertilization tpp1sa0011–/– mutants had higher levels of SCMAS throughout the embryo, including within the eye, head, spinal cord, and prominently within the muscle fibres compared with wild-type siblings (Fig. 4A–D). We were concerned whether such ubiquitous signal could be background histofluorescence or due to accumulation of autofluorescent storage material (attributable to ceroid and lipofuscin accumulation) in tpp1sa0011–/– zebrafish so we performed the same experiment without the anti-SCMAS primary antibody. Images demonstrate that the SCMAS staining in the CNS and muscle is neither background nor autofluorescence (Fig. 4I–L). To ascertain if storage is intralysosomal, LysoTracker® Red was used to label lysosomes, demonstrating the presence of enlarged lysosomes throughout the CNS as well as in yolk cells (not shown), but not in the muscle, in 48 h post fertilization tpp1sa0011–/– zebrafish compared with wild-type siblings (Fig. 4E–H). These data demonstrate that storage of SCMAS is present throughout tpp1sa0011–/– mutants and that the storage material accumulation at 48 h post fertilization correlates with Tpp1 deficiency and the visible onset of neurodegeneration, but is not associated with detectable autofluorescence at this stage. However, lysosomal enlargement is predominantly seen in the CNS.

**Neuropathology in Tpp1 deficient zebrafish**

Selective neuronal loss in the cerebral cortex, cerebellum and retina is another hallmark of CLN2 disease pathology (Nardocci et al., 1995; Petersen et al., 1996; Autti et al., 1997a; Lavrov et al., 2002; Sleat et al., 2004; Chang et al., 2008, 2011; Cooper et al., 2011). As Tpp1 deficient embryos have a smaller head and eye, we examined their CNS for a neuronal deficit in comparison with wild-type siblings. A profound absence of HuC/D-positive differentiated neurons was observed in 48 h post fertilization tpp1sa0011–/– (Fig. 5A and E; n = 5 each) and tpp1 hu3587+/− SPL morpholino injected wild-type (Supplementary Fig. 2A and D) retina, optic tectum and cerebellum when compared with wild-type (n = 5) that was also evident at 72 h post fertilization (data not shown). We did not detect such overt loss of neurons in other structures including the thalamus, telencephalon and olfactory bulbs using this method.

**Figure 1 Continued**

Figure 1 Continued

| tpp1hu3587+/− carrier offspring was raised to adulthood and sequencing of exon 11 revealed that wild-type siblings (E), tpp1hu3587+/− heterozygous carriers (F) and tpp1hu3587−/− homozygous mutants (G) can survive to adulthood. Reverse transcriptase-PCR of tpp1 SPL morpholino injected wild-type zebrafish (H, Lane 1) confirmed mis-splicing of tpp1 messenger RNA transcript compared with the normal tpp1 transcript (H, Lane 2). (I) Proportion of wild-type to tpp1sa0011–/– mutants obtained over 10 crosses of tpp1sa0011+/− carriers shows autosomal recessive inheritance of the tpp1sa0011–/– phenotype. Mean percentage of tpp1sa0011–/– obtained was 28%. Western blotting (J) confirmed the near complete absence of 46kDa mature Tpp1 in tpp1sa0011–/– mutants as well as complete Tpp1 absence in tpp1 ATG morpholino injected 48 h post fertilization wild-type embryos. Tpp1 enzyme activity was measured for 90 min in 96 h post fertilization tpp1sa0011−/− mutants and wild-type sibling crude protein extracts using the Arg-Ala-Phe-ACC substrate (K, n = 7). Tpp1 activity was significantly diminished in tpp1sa0011−/− mutants compared to wild-type (P < 0.0001). Kaplan-Meier survival analysis (G), comparing wild-type, tpp1sa0011−/− and tpp1hu3587−/− embryos (15 of each), shows the probability of tpp1sa0011−/− mutant survival compared with wild-type siblings. tpp1sa0011−/− mutants started to die at 4 days post fertilization and all had died by 7 days post fertilization. The median survival was determined as 5 days post fertilization. All 15 wild-type siblings and tpp1hu3587−/− embryos survived beyond 7 days post fertilization at which point the experiment was concluded. Log rank (Mantel-Cox) test applied to the null hypothesis that there is no difference between survival functions (P < 0.0001). Black arrows = site of base pair change. hpf = hours post fertilization.
Figure 2. Similarity of phenotypes obtained after manipulation of tpp1. Bright-field images show wild-type siblings (A and B), \(tpp1^{sa0011}/-/\) larvae (C and D), \(tpp1^{hu3587}/-/\) (E and F), \(tpp1^{sa0011}+/+\) and \(tpp1^{hu3587}+/+\) cross (G), \(tpp1^{ATG}\) morpholino (H and I) and \(tpp1^{SPL}\) morpholino (J and K). All \(tpp1\) manipulations cause small eyes, a small head and loss of jaw compared with wild-type siblings. The offspring from a \(tpp1^{hu3587}+/+\) incross display a variably penetrant phenotype. At 48 h post fertilization offspring displayed a range of phenotypes from seeming wild-type to a typical \(tpp1\) mutant phenotype. Quantification of mean retinal area at several time points (L) shows significantly impaired retinal growth over time in \(tpp1^{sa0011}/-\) mutants compared with wild-type (unpaired t-test \(P<0.001\) at 48 h and 72 h post fertilization; \(P=0.01\) at 54 h post fertilization) \((n=10\) at 48 h post fertilization, \(n=4\) at both 54 h post fertilization and 72 h post fertilization). Dashed line indicates outline of the eye. Error bars = SEM; scale bar 300 \(\mu\)m. FB = forebrain; hpf = hours post fertilization; MO = morpholino; WT = wild-type.
Astrocytosis is also a significant and early pathological feature of CLN2 disease in the mouse model (Chang et al., 2008) and when we looked at glial fibrillary acidic protein (GFAP) expression (a marker of astrocytosis) we found localized GFAP immunoreactivity in the thalamus and cerebellum of 48 h post fertilization tpp1sa0011/C0/C0 zebrafish that was absent in wild-type siblings (Fig. 5B and F).

Apoptosis is evident in Tpp1 deficient zebrafish

The absence of neurons in specific parts of the CNS of tpp1sa0011/C0/C0 zebrafish could reflect cell death or a defect in the development of neurons. As TUNEL staining of CNS tissue obtained at autopsy from patients with CLN2 disease has

Figure 3 Tpp1 absence in tpp1sa0011/C0/C0 mutant retinal cells. Ten micrometre-thick retinal sections of 48 h post fertilization wild-type siblings (A and B) and tpp1sa0011/C0/C0 mutants (C and D) were immunolabelled with rabbit anti-TPP1. Tpp1 is expressed throughout the wild-type retina (A) with prominent expression in the ganglion cell layer, inner plexiform layer and the outer nuclear layer (B). In the tpp1sa0011/C0/C0 mutant retina (C), no obvious Tpp1 protein is observed (D) and the retinal architecture is abnormal owing to widespread pathology. BF = brightfield; GCL = ganglion cell layer; INL = inner nuclear layer; IPL = inner plexiform layer; ONL = outer nuclear layer; OPL = outer plexiform layer; n = 6 sections each for wild-type and tpp1sa0011/C0/C0 mutant. Scale bars: brightfield = 100 μm; immunofluorescence = 10 μm.
Figure 4  Storage material accumulates in \( tpp1^{sa0071-/-} \) embryos. Forty-eight hours post fertilization wild-type siblings (A, B, E, F, I, I', J and J') and \( tpp1^{sa0071-/-} \) (C, D, G, H, K, K', L and L') were immunostained with rabbit SCMAS (A–D), LysoTracker® Red (E–H), or using the same protocol as for the SCMAS immunofluorescence, but without the primary antibody (I–K and I'–K'). No significant SCMAS staining observed in the head (A) or tail (B) of wild-type siblings. Extensive SCMAS storage is observed throughout \( tpp1^{sa0071-/-} \) mutants including within the retina and forebrain in the head (C), and muscle fibres in the tail (D). LysoTracker® Red staining revealed small punctate lysosomes in the retina, forebrain and hindbrain of wild-type siblings (E), with no significant staining in the tail (F). In \( tpp1^{sa0071-/-} \) embryos, lysosomes are greatly enlarged in the retina, forebrain and hindbrain (G) but not in the tail (H). Immunofluorescence performed without primary antibody shows no significant background immunofluorescence or autofluorescence in wild-type siblings (I and J) or \( tpp1^{sa0071-/-} \) (K and L). Brightfield images of tissues shown in I–L are shown in I'–L'. FB = forebrain; m = muscle fibres; R = retina; OV = otic vesicle; WT = wild-type. \( n = 6 \) each for wild-type and \( tpp1^{sa0071-/-} \) mutants. Scale bar = 100 µm.
Figure 5  Tpp1 knockdown induces early onset neurodegeneration. Anti-HuC/D (A and E), anti-GFAP (B and F), anti-phosphohistone 3 (PH3) (C and G) and anti-acetylated alpha tubulin (D and H) immunofluorescence was performed on 48 h post fertilization wild-type siblings (A–D) and tpp1sa0011/C0/C0 mutants (E–H). HuC/D reveals differentiated neurons and shows a missing cerebellum, retina and optic tectum in tpp1sa0011/C0/C0 mutants (E) compared with wild-type siblings (A). GFAP labels few glia in the brain of wild-type siblings (B), but is evident in the thalamus and cerebellum of tpp1sa0011/C0/C0 mutants (F). Phosphohistone 3 labels cell proliferation and shows impaired proliferation in the CNS of tpp1sa0011/C0/C0 mutants (G) compared with wild-type siblings (C). Acetylated alpha-tubulin labels axons and reveals defects within the optic nerve, posterior commissure and trochlear nerve in tpp1sa0011/C0/C0 (H) compared with wild-type siblings (D). AC = anterior commissure; C = cerebellum; FB = forebrain; ON = optic nerve; OT = optic tectum; PC = posterior commissure; R = retina; SOT = supraoptic tract; TPOC = tract of the post optic commissure; T = thalamus; IV = trochlear nerve. (n = 5 for wild-type and tpp1sa0011/C0/C0 mutants for each stain). Scale bar = 100 μm. To quantify cell proliferation in specific CNS regions of wild-type (continued)
previously shown selective neuronal cell death in the cerebral cortex, cerebellum and retina (Lane et al., 1996; Kurata et al., 1999; Mitchison et al., 2004), we examined cell death in \textit{tpp1}^{sa0011}\textsuperscript{+/-} zebrafish using the TUNEL assay. Examination of embryos derived from \textit{tpp1}^{sa0011}\textsuperscript{+/-} heterozygote in-crosses at 24 h, 30 h and 42 h post fertilization did not reveal TUNEL-positive bodies greater than expected for the particular stage of development in any of the embryos (data not shown) (Cole and Ross, 2001). However, when compared with wild-type siblings from 48 h to 54 h post fertilization, mutants possessed a large number of TUNEL-positive apoptotic bodies selectively localized in the retina, optic tectum, cerebellum and throughout the spinal cord (Fig. 6A–H). However by 72 h post fertilization, although there were numerous apoptotic bodies in the skin throughout the length of the \textit{tpp1}^{sa0011}\textsuperscript{+/-} mutant, only a few were seen within neural tissue. We quantified the TUNEL positive bodies in the retina, forebrain, mid/hindbrain and the spinal cord (somites 14–20) from 48 h to 72 h post fertilization (\(n = 4\) each, wild-type and \textit{tpp1}^{sa0011}\textsuperscript{+/-} mutant). The Mann–Whitney U-test, comparing wild-type and \textit{tpp1}^{sa0011}\textsuperscript{+/-} embryos at 48 h and 54 h post fertilization gave \(P < 0.0286\) and at 72 h post fertilization \(P = 1\) (Fig. 6I). Therefore Tpp1 deficient zebrafish show profound early onset, localized cell death in a subset of CNS regions homologous to some of those affected in human CLN2 disease. Moreover, increased cell death in \textit{tpp1}^{sa0011}\textsuperscript{+/-} mutants is first visible at 48 h post fertilization consistent with the onset of morphological abnormalities and also suggests that apoptosis is an early, not late, feature of CLN2 disease. The combined pathological features of \textit{tpp1}^{sa0011}\textsuperscript{+/-} zebrafish indicate that it is a good model for human CLN2 disease. We next sought to examine \textit{tpp1}^{sa0011}\textsuperscript{+/-} zebrafish for novel defects that have not yet been described in other models of CLN2 disease.

## Reduction of cell proliferation in the zebrafish \textit{tpp1}^{sa0011}\textsuperscript{+/-} central nervous system

Altered CNS cell proliferation has not yet been examined in animal models of CLN2 disease despite being implicated in other forms of neuronal ceroid lipofuscinoses including CLN3 disease, juvenile (Kay et al., 2006; Vantaggiato et al., 2009; Weimer et al., 2009). We investigated if the marked absence of differentiated neurons correlated with impaired cell proliferation or if proliferation was enhanced as an attempt to repair the CNS. Phosphohistone H3 labelling of Tpp1 deficient zebrafish was performed between 48 h post fertilization and 72 h post fertilization (\(n = 5\) each) and the number of proliferative cells were quantified in four different regions [retina, forebrain, mid-hindbrain and spinal cord (somite 14–20)] of wild-type and \textit{tpp1}^{sa0011}\textsuperscript{+/-} zebrafish at each stage. There was no difference in the level of proliferative neuronal precursors before 48 h post fertilization (data not shown). At 48 h post fertilization cell proliferation within the \textit{tpp1}^{sa0011}\textsuperscript{+/-} zebrafish (Supplementary Fig. 2C and F) retina, forebrain, midbrain-hindbrain boundary and spinal cord was significantly reduced compared with wild-type zebrafish. The difference in proliferation of cells between wild-type and \textit{tpp1}^{sa0011}\textsuperscript{+/-} retina, forebrain and midbrain-hindbrain boundary, but not spinal cord, was significant at 54 h post fertilization and 72 h post fertilization (Fig. 5I–L). These data show that reduced cell proliferation is extensive from phenotypic onset to beyond 72 h post fertilization, lasting longer than apoptosis in \textit{tpp1}^{sa0011}\textsuperscript{+/-} zebrafish.

## Axon tracts are disrupted in Tpp1 deficient zebrafish

Axonal disorganization and white matter tract degeneration is widespread both in the CNS of patients with CLN2 disease (Goebel et al., 1999), as well as in the murine model of CLN2 disease (Sleat et al., 2004; Cabrera-Salazar et al., 2007). Despite white matter being reduced in patients with CLN2 disease and animal models, it is not known whether these defects are a consequence of neuronal loss of whether there are primary defects in axon growth, guidance and fasciculation. In addition, by 24 h post fertilization zebrafish possess the primary axonal (scaffold) tracts including the anterior commissure, supraoptic tract, the tract of the post optic commissure, posterior commissure and medial longitudinal fasciculus, which further mature by 48 h post fertilization (Wilson et al., 1990). The \textit{tpp1}^{sa0011}\textsuperscript{+/-} zebrafish model is therefore well suited to investigating axon tract formation in CLN2 disease. Neurodevelopmental defects of axon guidance in Tpp1 deficient embryos were seen in some of these tracts when stained with an antibody to acetylated alpha-tubulin. Within both 48 h post fertilization \textit{tpp1}^{sa0011}\textsuperscript{+/-} zebrafish (Fig. 5D and H) and \textit{tpp1} SPL morpholino injected wild-type embryos (Supplementary Fig. 2B and E) the primary axon tracts begin to look disorganized compared with wild-type siblings. The posterior commissure is highly de-fasciculated from the optic tectum to the anterior tegmentum, and the optic nerve is almost completely absent with no fibres apparently reaching the optic tectum after crossing at the optic chiasm. In addition, the trochlear nerve, which lies...
Figure 6  Neurodegeneration is evident in \( tpp^{sa0011-/-} \) mutants in comparison with wild-type siblings. The TUNEL assay was performed in 1-phenyl-2-thiourea (PTU)-treated embryos to detect apoptosis at various time periods and apoptotic bodies were quantified over several equivalent planes of foci in each embryo. At 48 h post fertilization no apoptotic bodies are detected in the eye or CNS tissue of wild-type siblings (A and G) compared with \( tpp^{sa0011-/-} \), which has a large number of apoptotic bodies localized in the retina, optic tectum, hindbrain and rostral portion of the spinal cord (D and H). At 54 h post fertilization few apoptotic bodies are present in the wild-type sibling retina and apoptotic bodies accumulate in large cells on the surface of the forebrain and midbrain regions (B), compared with \( tpp^{sa0011-/-} \) mutants, which has a slightly increased number of apoptotic bodies (E). At 72 h post fertilization the wild-type sibling has an increased number of positively stained cells outside the CNS (C), as do \( tpp^{sa0011-/-} \) mutants (F). Quantification of TUNEL-positive bodies in wild-type siblings versus \( tpp^{sa0011-/-} \) mutants (I) demonstrates a significantly increased median number of total apoptotic bodies present in the \( tpp^{sa0011-/-} \) CNS versus wild-type siblings at 48 h and 54 h post fertilization but not at 72 h post fertilization. Mann–Whitney U test, \( P = 0.0286 \) at 48 h and 54 h post fertilization, whereas \( P = 1 \) at 72 h post fertilization (\( n = 4 \) at all time stages for both wild-type and mutant) (error bar = SEM). FB = forebrain; HB = hindbrain; hpf = hours post fertilization; OT = optic tectum; R = retina; SC = spinal cord; WT = wild-type. Scale bar = 100 \( \mu m \).
immediately rostral to the cerebellum, is completely absent in Tpp1 deficient zebrafish. Observations of later stages in 54 h and 72 h post fertilization tpp1<sup>Δa0011</sup>−/− larvae further confirm the complete absence of tectal fibres and trochlear nerve, with no sign of late onset growth (data not shown). Furthermore, alpha-tubulin staining at 42 h post fertilization, before the onset of an abnormal phenotype in tpp1<sup>Δa0011</sup>−/− embryos, did not show any defects in primary axon tract formation (data not shown). These results imply that primary axon tract formation in Tpp1 deficient zebrafish is not significantly impaired and axon loss is secondary to degenerative changes. The pathological phenotype of our tpp1<sup>Δa0011</sup>−/− zebrafish confirms severe neurodegenerative changes consistent with cases of human CLN2 disease. We subsequently decided to investigate functional of traits the tpp1<sup>Δa0011</sup>−/− larvae, particularly locomotion, in order to investigate the potential of this model for high-throughput automated drug screening.

Tpp1 deficient zebrafish have abnormal locomotion that can form the basis of high-throughput drug screening

We sought to characterize the locomotion defects in tpp1<sup>Δa0011</sup>−/− zebrafish in more detail with a view to finding parameters that could form the basis of an automated assay suitable for high-throughput drug discovery. First we analysed the touch-evoked escape response. Zebrafish develop mechanosensory neuromasts and are touch responsive as early as 24 h after fertilization (Ghysen and Dambly-Chaudiere, 2004). Furthermore, by 48 h post fertilization a single stimulus to the head or tail is sufficient to evoke a highly characteristic motile response consisting of alternating C-shaped body movements, propelling the fish in a straight line (Brustein et al., 2003). tpp1<sup>Δa0011</sup>−/− embryos 48 h post fertilization and older were less responsive to touch than wild-type siblings and after repeated prodding with a pipette tip, responded with circular motion due to their curved body (data not shown).

Secondly, we observed free-swimming locomotion of tpp1<sup>Δa0011</sup>−/− mutants and found movement that may be related to seizure activity. Wild-type siblings at 72 h post fertilization and older were less responsive to touch than their wild-type siblings. Ethovision XT (Noldus) software was then used to quantitatively assess several behavioural parameters of 96 h post fertilization tpp1<sup>Δa0011</sup>−/− larvae compared with wild-type siblings at 96 h post fertilization which usually move an appreciable amount. The mean distance moved, velocity and proportion of time spent in motion by wild-type in a 170 s recording period was significantly greater (Mann-Whitney U, P = 0.0023, 0.0023 and 0.0012, respectively) in wild-type siblings than tpp1<sup>Δa0011</sup>−/− larvae (Fig. 8B–D). Furthermore the number of movement bouts (in this case averaged over 5 s with a start velocity of 0.4 mm/s and a stop velocity of 0.2 mm/s) was used to assess darting behaviour in wild-type and tpp1<sup>Δa0011</sup>−/− larvae. The frequency of movement bouts and mean distance moved during each movement bout were significantly lower in tpp1<sup>Δa0011</sup>−/− larvae compared with wild-type (Mann–Whitney U, P = 0.0042 and 0.0047, respectively) (Fig. 8E and F) whereas no significant differences in movement bout velocity were detected (data not shown).

Discussion

This study demonstrates that zebrafish can be used to model CLN2 disease. This is an early onset, autosomal recessive condition in which affected patients are homozygous for a mutation in the CLN2 disease-causing gene (TPP1) causing reduced or absent TPP1 enzyme activity (Sleat et al., 1997). Consistent with this, approximately one-quarter of all offspring obtained from zebrasibich tpp1<sup>Δa0011</sup>−/− display an abnormal early onset neurodegenerative phenotype and genotyping confirms the presence of an in-frame premature stop codon in exon 3 of the tpp1 gene. The most common mutations in CLN2 disease are null (exon 6: R208X in 58 families) or frameshift (intron 5: IVS5-1G>C in 67 families) whereas early TPP1 mutations, including the null mutation Q66X in exon 3, affect only a few families (Kousi et al., 2012) (www.ucl.ac.uk/ncl). Nonetheless the effect of these common mutations would result in an absence of TPP1 due to early degradation of the mis-formed protein, similar to the lack of Tpp1 in zebrafish. Therefore tpp1<sup>Δa0011</sup>−/− zebrafish are a representative stable mutant model of most cases of CLN2 disease.

Importantly, Tpp1 activity does not appear to be completely absent in tpp1<sup>Δa0011</sup>−/− as predicted by the genotype. This could be due to a small amount of non-specific Arg-Ala-Phe-ACC breakdown owing to the use of total protein from whole zebrafish for the enzyme assay. However, it is just as likely that the small amount of full-length Tpp1 we detected by western blot with protein from tpp1<sup>Δa0011</sup>−/− zebrafish is able to break down the substrate. This small amount of full length Tpp1 found in tpp1<sup>Δa0011</sup>−/− could be due to either perdurance of maternally-deposited protein, translation of maternally-deposited transcript or stop codon read-through. In support of this, others have
Figure 7  Locomotion is abnormal in 72 h post fertilization \textit{tpp}\textsuperscript{1\textsubscript{iso011}}\textsubscript{--/} mutants. Motion tracking plots reveal that at 72 h post fertilization \textit{tpp}\textsuperscript{1\textsubscript{iso011}}\textsubscript{--/} mutants (A, left) appear to have increased movement compared to wild-type siblings (A, right). This was quantitatively assessed using Ethovision XT software analysis of 20 min motion movies (Table 1; \(n=8\) wild-type siblings and 8 \textit{tpp}\textsuperscript{1\textsubscript{iso011}}\textsubscript{--/} at 72 h post fertilization). Panel B demonstrates that the criteria used to reveal movement bouts picks up periods of prolonged locomotion.

Table 1  Quantitative assessment of 20 min motion videos using Ethovision XT software

<table>
<thead>
<tr>
<th></th>
<th>Wild-type sibling</th>
<th>\textit{tpp}\textsuperscript{1\textsubscript{iso011}}\textsubscript{--/}</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total distance moved (mm)</td>
<td>182.553</td>
<td>969.143</td>
<td>0.0003*</td>
</tr>
<tr>
<td>Mean movement velocity (mm/s)</td>
<td>0.165</td>
<td>0.812</td>
<td>0.0019*</td>
</tr>
<tr>
<td>Maximum movement velocity (mm/s)</td>
<td>46.646</td>
<td>42.511</td>
<td>0.6454</td>
</tr>
<tr>
<td>Total time spent moving (s)</td>
<td>189.917</td>
<td>596.795</td>
<td>0.065</td>
</tr>
<tr>
<td>Frequency of movement bouts</td>
<td>4.5</td>
<td>24.5</td>
<td>0.02*</td>
</tr>
<tr>
<td>Duration of movement bouts (s)</td>
<td>5.804</td>
<td>54.375</td>
<td>0.0281*</td>
</tr>
</tbody>
</table>

\(n=8\) wild-type siblings and 8 \textit{tpp}\textsuperscript{1\textsubscript{iso011}}\textsubscript{--/} at 72 h post fertilization.

\(*=P<0.05.*
reported that the mature protein has a half-life of 20 h (Golabek et al., 2003), suggesting that maternal contribution could still be present at 48 h post fertilization, the age of the embryos used for western blot analysis. These results further imply that even a small amount of Tpp1 activity may be insufficient to prevent neurodegeneration in early developing zebrafish.

The genotype-phenotype correlation of $tpp1^{sa0011/-}/C0$ zebrafish is further strengthened by observing $tpp1^{sa0011/-}$ and $tpp1^{hu3587+/}$ inter-cross offspring. $tpp1^{sa0011/-}$ mutants are only mildly affected compared with $tpp1^{sa0011/-}$ mutants, with few developing early onset neurodegeneration and most reaching reproductive maturity. However, $tpp1^{sa0011/-}/tpp1^{hu3587-}$ trans-heterozygotes have a similar phenotype to $tpp1^{sa0011/-}$ mutants albeit with mild retinal degeneration and delayed disease onset of ~24 h. Finally, genotype-phenotype correlation of $tpp1^{sa0011/-}$ mutants was further established by morpholino knockdown experiments targeting the $tpp1$ gene. Injection of two different morpholinos in wild-type embryos resulted in a similar phenotype to $tpp1^{sa0011/-}$ mutants thereby implying a clear link between $tpp1$ gene defect, and a concomitant lack of Tpp1 activity, with early onset neurodegeneration.

The consequence of absent TPP1 is the build up of storage material inside lysosomes (Ezaki et al., 1999, 2000; Ezaki and Kominami, 2004). In human brain biopsy samples and leucocyte culture the accumulation of subunit c of mitochondrial ATP synthase (SCMAS) has been shown by immunohistochemistry (Hall et al., 1991; Palmer et al., 1992). We have demonstrated the ubiquitous storage of SCMAS in $tpp1^{sa0011/-}$ mutants throughout the body, most prominently in the CNS and muscles. Furthermore, MRI scans of patients with CLN2 disease show selective degeneration in the cerebral cortex, cerebellum and retina with the basal ganglia relatively preserved (Autili et al., 1997b;
D’Incerti, 2000). Consistent with these observations, the optic tectum, cerebellum and retina lack mature neurons in 48 h post fertilization tpp1\(^{sa0011/-}\) and we have shown this to be mediated by a combination of apoptotic cell death and aberrant cell proliferation well before these fish die. In wild-type zebrafish, the number of apoptotic bodies in the CNS remains low at 48, 54 and 72 h post fertilization, whereas the number of apoptotic bodies in tpp1\(^{sa0011/-}\) zebrafish is much greater than in wild-type at 48 and 54 h post fertilization, but similar to wild-type at 72 h post fertilization. This demonstrates an early increase in apoptosis in tpp1\(^{sa0011/-}\). In contrast, proliferation in the CNS of wild-type zebrafish is greatest at 48 and 54 h post fertilization and there is relatively little at 72 h post fertilization. In tpp1\(^{sa0011/-}\) zebrafish, proliferation is reduced at all stages assayed, demonstrating a sustained reduction in proliferation, predominantly affecting the retina and around the midbrain-hindbrain boundary where the cerebellum is developing. The role of apoptosis, however, remains controversial in CLN2 disease despite several studies showing TUNEL-positive bodies in the CNS of patients, molecular evidence for upregulation of apoptotic factors has not been forthcoming (Kurata et al., 1999; Kim et al., 2009). Additionally, it is likely that necrosis and autophagy may also play a role in mediating cell death (Mitchison et al., 2004). The zebrafish CLN2 disease model presented here thus provides a useful system for further investigating the various mechanisms mediating cell death in CLN2 disease. In addition, the majority of the specific regions that are affected in CLN2 disease, late infantile are prominently targeted in tpp1\(^{sa0011/-}\) leading to deficits representative of the human condition. However, we did not detect neuronal loss in the thalamus and telencephalon but it is possible that more sensitive methods, such as stereology, would demonstrate that these regions are also affected. Interestingly, Tpp1 (previously known as Cln2) mutant mice do not suffer from visual problems or retinal degeneration even up until late stages of the disease (Sleat et al., 2004), so in this case the zebrafish model appears relatively more representative of the human condition.

The phenotype manifested in the zebrafish is extremely severe and rapidly progressive which may reflect their rapid external development and relatively simple brain structure as well as overall shorter lifespan (Kimmel, 1993; Kimmel et al., 1995). The early timing of the pathology manifested in tpp1\(^{sa0011/-}\) shows the critical requirement of Tpp1 for zebrafish neurodevelopment. The highly defasciculated posterior commissure in 48 h post fertilization tpp1\(^{sa0011/-}\) and disorganized or absent trochlear nerve and tectal projections indicate a localized defect in mid-hindbrain axon guidance that is probably secondary to loss of neurons either resulting directly in a loss of axons or indirectly in a lack of guidance cues. Furthermore phosphohistone H3 staining demonstrates a loss of proliferative cells at the onset of secondary neurogenesis (Mueller and Wullimann, 2003) in 48 h post fertilization tpp1\(^{sa0011/-}\) embryos. Thus it could be that there are few mature neurons present in the regions of the affected CNS and non-proliferating neural progenitors are subjected to apoptosis, perhaps owing to an inability to divide or that mature and proliferative cells are equally affected. In addition, the earliest Tpp1 expression in human foetal samples was observed at 12 weeks gestation and some neurons in the cerebral cortex expressed TTP1 only post-natally with TTP1 expression reaching adult levels at 2 years of age (Kida et al., 2001). Thus the onset of TTP1 expression in humans is more gradual throughout development. Although this may explain the delay in manifestation of pathological consequences of TTP1 deficiency in humans, it may also be explained by continued maternal contribution of TTP1 protein across the placenta until birth. The zebrafish develops externally and hence a placental source of maternal contribution does not exist. Lastly, humans express TTP2, a highly related enzyme, which is not found in zebrafish. Therefore the presence of TTP2 may also contribute to the later onset of CLN2 disease in humans compared with zebrafish. Nonetheless, the zebrafish CLN2 disease model can be used to further investigate the early pathological consequences of TTP1 deficiency.

Zebrafish larvae are highly motile displaying a variety of stereotypical movement behaviours from early development (Brustein et al., 2003). From as early as 17 h post fertilization the embryonic zebrafish undergo spontaneous tail coiling, followed by the development of the touch response by 21 h post fertilization and finally the first swimming behaviour is elicited at 27 h post fertilization (Brustein et al., 2003). Furthermore, three typical swimming behaviours are elicited between 48 h and 96 h post fertilization including cyclic swimming, slow starts and fast-startle responses, which all depend upon an upright posture being adopted by the zebrafish (Müller and van Leeuwen, 2004). By 96 h post fertilization the zebrafish larva is freely swimming, able to change direction spontaneously and swim towards targets (Granato et al., 1996).

In contrast with wild-type zebrafish, the tpp1\(^{sa0011/-}\) larvae have impaired escape response by 48 h post fertilization, abnormal, seizure-like motility by 72 h post fertilization and impaired locomotion by 96 h post fertilization. Most tpp1\(^{sa0011/-}\) larvae have a permanently curved body. Some larvae are able to flick their tail to the left and the right and swim in fairly straight lines but most larvae are incapable of making the alternate C-shaped contractions that are required for motion in a straight line. As a result, tpp1\(^{sa0011/-}\) zebrafish swim in circles and have a restricted range of motion. At 72 h post fertilization tpp1\(^{sa0011/-}\) zebrafish exhibit activity that may be seizure related, although it will require electrophysiological recordings to determine if these are epileptiform events. Similarly, the repetitive twitching may represent myoclonus and this needs conformation using electromyography. By 96 h post fertilization, nearly all ability to move is lost in tpp1\(^{sa0011/-}\) mutants, reflecting the loss of mobility seen in patients over time. The motor consequences of Tpp1 deficiency in tpp1\(^{sa0011/-}\) larvae therefore closely reflect the human CLN2 disease condition.

In summary, tpp1\(^{sa0011/-}\) zebrafish do recapitulate many features of CLN2 disease. A comparison of the currently available CLN2 disease models and the extent to which they demonstrate the specific features of CLN2 disease, presented in Table 2, which shows that no model has yet been shown to have all features of the disease. Although the study of disease mechanisms and development of experimental therapies will rely on the use of all three models, the zebrafish has a unique role in providing a platform for drug discovery.
Table 2  A comparison of the zebrafish tpp1sa0011 mutant with the hallmarks of CLN2 disease, late infantile and the mouse and long-haired dachshund dog models of CLN2 disease

<table>
<thead>
<tr>
<th>Clinical signs</th>
<th>Human CLN2 disease, late infantile</th>
<th>Mouse Cln2 (Tpp1) mutants</th>
<th>Long-haired Dachshund</th>
<th>Zebrafish tpp1sa0011</th>
</tr>
</thead>
<tbody>
<tr>
<td>Movement changes over time</td>
<td>Ataxia, seizures, myoclonus, loss of motor function</td>
<td>Motor deficits, tremors, seizures (infrequent), hyperactivity</td>
<td>Ataxia, myoclonus, seizures, hyperactivity</td>
<td>Bouts of seizure-like excessive movement, loss of motor function</td>
</tr>
<tr>
<td>Vision</td>
<td>Blindness</td>
<td>Not described</td>
<td>Blindness</td>
<td>Reduced</td>
</tr>
<tr>
<td>Lifespan</td>
<td>Reduced</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pathology</td>
<td>Atrophy in cortex/cerebellum, retina</td>
<td>Atrophy in thalamocortical and cerebellar systems, less in retina</td>
<td>Atrophy in cerebellum and retina, not described in cortex</td>
<td>Atrophy in optic tectum, cerebellum and retina,</td>
</tr>
<tr>
<td>Brain/retina</td>
<td>Reduced</td>
<td></td>
<td>Reduced</td>
<td></td>
</tr>
<tr>
<td>White matter</td>
<td>Reduced</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ventricles</td>
<td>Enlarged ventricles</td>
<td>Not described</td>
<td>Enlarged ventricles</td>
<td>Reduced</td>
</tr>
<tr>
<td>Microglia</td>
<td>Microglial activation</td>
<td>Astrocitosis</td>
<td>Astrocytosis</td>
<td>Not described</td>
</tr>
<tr>
<td>Astrocytes</td>
<td>Astrocitosis</td>
<td>Accumulation</td>
<td>Astrocytosis</td>
<td>No accumulation</td>
</tr>
<tr>
<td>Ceroid and lipofuscin (autofluorescence)</td>
<td>SCMAS accumulation</td>
<td>Not described</td>
<td>SCMAS accumulation</td>
<td>Not described</td>
</tr>
<tr>
<td>SCMAS</td>
<td></td>
<td>Periodic acid-Schiff-positive storage bodies</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Periodic acid-Schiff</td>
<td>SCMAS storage bodies</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Ultrastructure</td>
<td>Curvilinear profiles</td>
<td>Curvilinear profiles</td>
<td>Curvilinear profiles</td>
<td>Curvilinear profiles</td>
</tr>
</tbody>
</table>

Reference: Mole et al., 2011.

Conclusions and future work

The rapidly progressive pathological-behavioural CLN2 disease phenotype in tpp1sa0011-/- zebrafish combined with the ability to automate assessment of movement provides the ideal platform for high-throughput drug screening with the aim of developing therapeutic or palliative compounds for CLN2 disease. Firstly, individual zebrafish embryos can be placed in a single well of a 96-well plate and compounds applied at various time-points and concentrations (Goldsmith, 2004; Barros et al., 2008; Rihel et al., 2010). Furthermore, movement behaviour at 48 h post fertilization and 96 h post fertilization as well as enhanced survival prospects can be used as a marker for phenotypic improvement in tpp1sa0011-/- mutants.

Since the tpp1sa0011-/- model possesses an early null mutation, premature termination codon read-through compounds could be tested such as those that have entered phase 3 trials for Duchenne muscular dystrophy and cystic fibrosis. Previous research has suggested that 10–15% of the normal level of TPP1 would be sufficient to prevent neuronal ceroid lipofuscinoses [reviewed in Sleat et al. (2008b)], so this could be a promising avenue to pursue. In addition, screening of FDA-approved and other compound libraries, could help identify anti-apoptotic, anti-spasticity and survival enhancing drugs that improve function and reduce morbidity in patients with CLN2 disease. Any efficacious and safe therapy identified can be further validated in higher vertebrates such as existing TPP1 deficient mouse and canine models before trial in humans.

There are several aspects of this model that remain to be characterized, or have only been partially characterized, that could provide further mechanistic insights or markers for drug testing. The potential of the zebrafish as a model organism for human disease is just beginning to be realized. Our zebrafish CLN2 disease model provides the first stable genetic zebrafish model of a childhood neurodegeneration and epilepsy and provides proof of principle that zebrafish can be used to model such disorders.

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Supplementary material
Supplementary material is available at Brain online.

References


