doi:10.1093/brain/awp108 Brain 2009: 132; 1613–1623 | 1613

BRAIN
A JOURNAL OF NEUROLOGY

Complex I deficiency and dopaminergic neuronal cell loss in *parkin*-deficient zebrafish (*Danio rerio*)

Laura Flinn,[1,2] Heather Mortiboys,[2] Katrin Volkmann,[3] Reinhard W. Köster,[3] Phillip W. Ingham[1] and Oliver Bandmann[1,2]

1 MRC Centre for Biomedical and Developmental Genetics, University of Sheffield, Sheffield, UK
2 Academic Neurology Unit, Medical School, University of Sheffield, Sheffield, UK
3 Helmholtz-Centre Munich, Institute of Developmental Genetics, Ingolstädter Landstrasse 1, 85764 Neuherberg-Munich, Germany

Correspondence to: O. Bandmann,
Academic Neurology Unit,
Medical School, University of Sheffield,
Beech Hill Road,
Sheffield S10 2RX, UK
E-mail: o.bandmann@sheffield.ac.uk

Currently, only symptomatic therapy is available for Parkinson's disease. The zebrafish is a vertebrate animal model ideally suited for high throughput compound screening to identify disease-modifying compounds for Parkinson's disease. We have developed a zebrafish model for Parkin deficiency, the most commonly mutated gene in early onset Parkinson's disease. The zebrafish Parkin protein is 62% identical to its human counterpart with 78% identity in functionally relevant regions. The *parkin* gene is expressed throughout zebrafish development and ubiquitously in adult zebrafish tissue. Abrogation of Parkin activity leads to a significant decrease in the number of ascending dopaminergic neurons in the posterior tuberculum (homologous to the substantia nigra in humans), an effect enhanced by exposure to MPP+. Both light microscopic analysis and staining with the pan-neuronal marker HuC confirmed that this loss of dopaminergic neurons is not due to general impairment of brain development. Neither serotonergic nor motor neurons were affected, further emphasizing that the effect of *parkin* knockdown appears to be specific for dopaminergic neurons. Notably, *parkin* knockdown zebrafish embryos also develop specific reduction in the activity of the mitochondrial respiratory chain complex I, making this the first vertebrate model to share both important pathogenic mechanisms (i.e. complex I deficiency) and the pathological hallmark (i.e. dopaminergic cell loss) with human *parkin*-mutant patients. The zebrafish model is thus ideally suited for future drug screens and other studies investigating the functional mechanisms underlying neuronal cell death in early onset Parkinson's Disease. Additional electron microscopy studies revealed electron dense material in the t-tubules within the muscle tissue of *parkin* knockdown zebrafish. T-tubules are rich in L-type calcium channels, therefore our work might also provide a tentative link between genetically determined early onset Parkinson's disease and recent studies attributing an important role to these L-type calcium channels in late onset sporadic Parkinson's disease.

Keywords: Parkinson's disease; mitochondria

Abbreviations: Dpf = day post-fertilization; Hpf = hour post-fertilization; MO = morpholino; MPP+ = 1-methyl-4-phenylpyridinium; SNP = single nucleotide polymorphisms

Received December 9, 2008. Revised March 5, 2009. Accepted March 19, 2009. Advance Access publication May 12, 2009

Introduction

Autosomal recessively inherited, homozygous or compound heterozygous mutations in the *parkin* gene on chromosome 6q25–27 are the most common identifiable genetic cause for early onset parkinsonism (Kitada *et al*., 1998). There is also an ongoing debate whether a single heterozygote *parkin* mutation may be a susceptibility factor for late onset Parkinson's disease (Kay *et al*., 2007; Klein *et al*., 2007; Langston *et al*., 2007). The *parkin* gene (PARK2) encodes a ubiquitin E3 ligase (Shimura *et al*., 2000). Impaired mitochondrial function is currently seen as an important mechanism leading to neuronal cell loss in both genetically determined forms of early onset Parkinson's Disease and late onset, apparently sporadic Parkinson's disease (Schapira, 2008). Data on mitochondrial function in post-mortem brain tissue of patients with two *parkin* mutations are not available yet, but we and others have identified isolated complex I deficiency in peripheral tissue of *parkin*-mutant patients (Muftuoglu *et al*., 2004; Mortiboys *et al*., 2008). The results from the different post-mortem studies of *parkin*-mutant brains are somewhat variable, but share the core histopathological feature of neuronal cell death in the ascending nigrostriatal dopaminergic system with sporadic Parkinson's disease (Cookson *et al*., 2008).

Zebrafish (*Danio rerio*) are vertebrates and therefore more closely related to humans than other model organisms such as *Drosophila* or *Caenorhabditis elegans*. They have a short generation time (3 months) and breed prodigiously (hundreds of offspring per female per week). Embryos develop externally, can readily be manipulated genetically and are transparent. Micro-injection of morpholino (MO) antisense oligonucleotides into zebrafish embryos at the single cell stage can be used to suppress the translation of a particular gene transiently (Ekker and Larson, 2001). The dopaminergic (DA) system is well characterized in both embryos and the adult zebrafish. Dopaminergic neurons are first detected at ~19-h post-fertilization (hpf) in a cluster of cells in the posterior tuberculum of the ventral diencephalon. These neurons represent the dopaminergic system ascending to the striatum, comparable to the nigrostriatal system in humans (Holzschuh *et al*., 2001; Rink and Wullimann, 2001, 2002*b*). Zebrafish homologues with typically widespread expression pattern already at early stages of embryonic development have been identified for several firmly established Parkinson's disease genes, namely *alpha-synuclein*, *PINK1* and *DJ-1* (Bretaud *et al*., 2007; Anichtchik *et al*., 2008; Flinn *et al*., 2008; Sun and Gilter, 2008).

We have identified a zebrafish orthologue of the human *parkin* gene, analysed its expression and used the MO antisense strategy to determine the effect of Parkin deficiency in zebrafish embryos. *parkin* knockdown (k/d) results in impaired mitochondrial function without concomitant abnormal mitochondrial morphology. *parkin* knockdown also leads to selective loss of dopaminergic neurons with increased susceptibility to the Parkinson's disease neurotoxin 1-methyl-4-phenylpyridinium (MPP+, the active metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, MPTP) without impairment of overall brain development and sparing of other neuronal sub-populations such as motor neurons which are also unaffected in human Parkinson's disease patients. Intriguingly, EM analysis of muscle tissue in *parkin* knockdown zebrafish suggests protein aggregation in transverse (t)-tubules. T-tubules are invaginations of the surface membrane which are of crucial relevance for the calcium (Ca^{2+}) homeostasis of the muscle tissue (Brette and Orchard, 2007). Of note, they are particularly rich in L-type Ca^{2+} channels. Our data therefore suggest a tentative link between mechanisms leading to early onset Parkinson's Disease and impaired Ca channel function which has recently been implicated in late onset, sporadic Parkinson's disease (Chan *et al*., 2007).

Materials and Methods

Fish strains and maintenance

The London wild-type (LWT) strain of zebrafish was used for all experiments. Embryos were collected after natural spawning, staged according to standard criteria, and synchronously raised at 28.5°C. All zebrafish husbandry and experimental procedures were performed in accordance with the UK. Animals (Scientific Procedures) Act.

Isolation and sequencing of zebrafish parkin

Human Parkin protein sequence (accession number NP_004553) was blasted to the zebrafish genome using Ensembl (http://www.ensembl.org/Danio_rerio/blastview). The conceptual translation product of a single annotated transcript (ENSDARG00000021555, zgc:112390; accession number NP_001017635) was identified as the top match. The predicted zebrafish protein sequence was aligned with the human protein using ClustalX (www.clustal.org), and the predicted transcript aligned with the zebrafish genome in order to determine exon–intron boundaries and splice site locations. To confirm the *in silico* data, the coding sequence of *parkin* was amplified from reverse-transcribed zebrafish cDNA, using overlapping *parkin*-specific primer sets and experimental sequences aligned with *in silico* predictions.

RT–PCR analysis of parkin mRNA expression

Total RNA was extracted from whole embryos at different stages of development and from various adult zebrafish organs, using Trizol reagent (Invitrogen, Paisley, UK). Up to 3 μg of total RNA per sample was used to synthesize first-strand cDNA using reverse transcriptase (Superscript II kit; Invitrogen). The resulting cDNA (2 μl) was amplified using primers for *parkin*, 5′-GCGAGTGTGTCTGAGCTGAA-3′ (forward) and 5′-CACACTGGAACACCAGCACT-3′ (reverse); for *β-actin*, 5′-AAGCAGGAGTACGA-TGAGTCTG-3′ (forward) and 5′-GGTAAACGCTTCTGGAATGAC-3′ (reverse) were used as a positive control to confirm consistent quality and quantity of cDNA.

MO-mediated knockdown of Parkin

Anti-sense oligonucleotides (MO, GeneTools LLC, Philomath, OR, USA) were designed by GeneTools to target the splice donor site of exon 9 and splice acceptor site of exon 10 of the zebrafish *parkin* transcript. The MO sequences were: e9i9 5′-TGATTTGGTTCT CTTACCCCACAGC-3′ and i9e10 5′-ACACAAACTGACA-CAAACAG CAAAT-3′. Control MOs with five mismatches were used in order to distinguish phenotypic effects specific to the knockdown of *parkin*

from the effects of non-specific MO toxicity. MOs were re-suspended in sterile water at 2 mM stock concentration. Immediately prior to injection, e9i9 and i9e10 MOs were diluted to 0.75 mM and 1% phenol red (Sigma, Poole, UK) added to monitor injection efficiency. MOs were injected into the yolk of one- to two-cell stage embryos. Both *parkin* and control MOs were injected at 1.5 μM final concentration. To confirm efficacy of the splice-blocking MOs, 2 μl of cDNA were used in PCR amplifications using primers 5′-ATCGCCTGCAC AGACATCAT-3′ (forward) and 5′-GAGCCGCTTCTTCATCTACAA-3′, spanning exons 6–11 of the *parkin* mRNA.

Mitochondrial respiratory chain function assays

Embryos harvested at 72 hpf (15–30 per sample) were homogenized in 200 μl buffer (250 mM sucrose, 20 mM HEPES, 3 mM EDTA, pH 7.5), then stored at −80°C until ready for use. All spectrophotometric assays were performed at 30°C in 200 μl final volume using a Fluostar Omega spectrophotometer/plate reader, samples were diluted to 0.7 mg protein/ml and subjected to three cycles of liquid N_2 freeze thawing. Complex I activity was determined by following the oxidation of nicotinamide adenine dinucleotide reduced disodium salt (NADH) at 340 nm with a reference wavelength of 425 nm ($\varepsilon = 6.22\ mM^{-1}\ cm^{-1}$). The assay buffer contained 25 mM KH_2PO_4, 5 mM $MgCl_2$, (pH 7.2), 3 mM KCN, 2.5 mg/ml BSA, 50 μM ubiquinone Q1, 2 μg/ml antimycin A and zebrafish homogenate, the reaction was started with 125 μM NADH and inhibited with 3 μg/ml rotenone. Complex II activity was measured by following the reduction of 2,6-dichlorobenzenone-indophenol sodium salt (2,6-DCPIP) at 600 nm ($\varepsilon = 19.2\ mM^{-1}\ cm^{-1}$). The assay buffer contained 25 mM KH_2PO_4, 5 mM $MgCl_2$, (pH 7.2), 3 mM KCN, 20 mM succinate and zebrafish homogenate and was incubated for 10 min before the addition of 50 μM 2,6-DCPIP, 2 μg/ml antimycin A and 3 μg/ml rotenone, the reaction was started with 50 μM ubiquinone Q1. The specific activity was normalized to that of citrate synthase.

Complex III was measured by following the reduction of cytochrome c at 550 nm with a reference wavelength of 580. The assay buffer contained 25 mM potassium phosphate, 5 mM $MgCl_2$, 3 mM KCN, 2.5 mg per ml BSA, 15 μM cytochrome c, 3 μg/ml rotenone and 50 μM ubiquinol-2. The non-enzymatic reduction of cytochrome c by ubiquinol-2 was measured for 1 min, after which *x* μl zebrafish homogenate was added and the reaction followed for a further 4 min, finally a few grains of ascorbate were added to maximally reduce the cytochrome c. The rates were calculated as a first order constant and the results are expressed as K/min/citrate synthase activity. Complex IV activity was measured by following the oxidation of reduced cytochrome c at 550 nm, with a reference wavelength of 580 nm. The assay buffer contained 20 mM potassium phosphate, (pH 7.2), 150 μg/ml *n*-dodecyl-β-D-maltoside, 15 μM reduced cytochrome c and mitochondrial extract. The reaction was followed for 3 min after which time a few grains of potassium ferricyanide were added to fully oxidize the cytochrome c. The rates were calculated as a first order constant and the results are expressed as K/min/citrate synthase activity.

Toxin exposure using MPP+

WT (uninjected), control MO-injected and *parkin* knockdown embryos were raised for 48 hpf before exposure to toxins. At 24 hpf, embryos were transferred in groups of 20 to 12-well plates containing E3 embryo media. At 48 hpf, media was replaced in the treatment groups with 3 mM MPP+ (Sigma, Poole, UK) in E3 embryo media. Propylthiouracil (Sigma) was used at 0.2 mM concentration to prevent the development of pigmentation in all experiments other than the behavioural analysis. Embryos were harvested at 72 hpf, fixed overnight at 4°C in 4% paraformaldehyde, then washed into 100% methanol and stored at −20°C. All experiments were undertaken in triplicate.

In situ hybridization for quantification of dopaminergic neurons

Whole-mount *in situ* hybridization was undertaken as previously described (Thisse and Thisse, 2008). Briefly, a digoxigenin-labelled antisense RNA probe was synthesized from pBS-tyrosine hydroxylase (TH) plasmid linearized by *Xho*I and transcribed using T3 RNA polymerase (Invitrogen, Paisley, UK). Embryos were fixed at 3-day post-fertilization (dpf) overnight in 4% paraformaldehyde in phosphate-buffered saline (PBS), then transferred to 100% methanol for storage at −20°C for at least 24 h before undergoing hybridization. After the hybridization procedure was performed as described (Thisse and Thisse, 2008), embryos were washed extensively in 0.1% Tween 20 in PBS, re-fixed in 4% paraformaldehyde in PBS, then transferred to 80% glycerol. Embryos were mounted in glycerol and TH-positive diencephalic neurons counted under a Zeiss microscope. In order to avoid introducing unintended bias, all embryos were coded and scored blind. The results are expressed as percentage of neurons present in WT embryos (uninjected), which were staged and treated in parallel with *parkin* knockdown embryos.

Combined in situ hybridization and antibody immunofluorescence

Digoxigenin-labelled antisense RNA probe was synthesized from pCRIItopo-aromatic *amino acid decarboxylase (AADC)* plasmid, linearized by *Xho*I and transcribed using Sp6 RNA polymerase (Invitrogen). A modified *in situ* hybridization protocol was performed in which the anti-digoxigenin-POD antibody (Roche) was co-incubated with anti-tyrosine hydroxylase (TH) primary antibody (Millipore, UK) at 1:1000 final concentration overnight at 4°C. Embryos were extensively washed, stained with TSA-Cy3 (Perkin Elmer, Beaconsfield, UK) for *AADC* detection, again washed and then incubated with goat anti-rabbit AlexaFluor 488 antibody (Invitrogen, 1:500) for detection of TH. Whole-mount embryos were imaged using an Olympus laser scanning confocal microscope. *Z*-stack images were generated by scanning through the brain using a 20× objective at an optimal image slice distance. All *Z*-stack images of control and *parkin* knockdown embryos were captured using identical settings, determined empirically for optimal imaging of control MO embryos.

Whole-mount antibody immunofluorescence

For whole-mount antibody immunofluorescence detection of neuronal HuC/HuD proteins, embryos at 30 hpf were manually dechorionated, fixed at 4°C in 4% paraformaldehyde in PBS overnight, then stored at −20°C in 100% methanol. Embryos were serially washed into water, acetone shocked for 7 min at −20°C, then blocked for 2 h in 1% bovine serum albumin, 2% sheep serum in PBS containing 1% DMSO, 0.1% Tween 20 and 0.5% Triton X-100 (PBDTT).

After blocking, embryos were incubated overnight at 4°C in blocking solution containing monoclonal 16A11 anti-Huc/D (Invitrogen) at 1:500 final concentration, followed by washing several times with PBDTT and incubation with secondary antibody (goat anti-mouse AlexaFluor 488, Invitrogen). Whole-mount embryos were imaged on an Olympus laser scanning confocal microscope as described. For visualization of Islet-1 positive motor neurons, primary anti-Islet1 antibodies (Developmental Studies Hybridoma Bank, IA, USA) were used in the same procedure (Higashijima *et al*., 2000).

Behaviour analysis of parkin knockdown embryos

Embryos were tested for behavioural consequences of *parkin* knockdown by measuring swimming behaviour at 5 dpf. WT and *parkin* knockdown embryos were raised in E3 and at 5 dpf screened for inflated swim bladder formation. Fully developed larvae were arrayed 1 per well in six-well plates containing 4.5 ml of E3 and placed on a light stage with both a transmitted and infrared light source. The larvae were allowed to acclimate to the dish for 5 min, and then recorded for 5 min using Ethovision Pro digital video recording to track movement (Tracksys, Nottingham, UK). All digital tracks were analysed for total distance moved, using an input filter of 0.06 cm as the minimum distance to be considered 'movement'. Each experiment was performed in triplicate, and the results expressed as mean total movement ± SEM.

Electron microscopic analysis

Larvae injected with parkin MO and WT larvae were fixed in 1× PBS containing 2% paraformaldehyde and 2% glutaraldehyde at 5 dpf. Embedding, sectioning, and image acquisition were undertaken as previously described (Rieger and Koester, 2007).

Statistical analysis

All experiments were undertaken in triplicate, each with $n = 10$ per treatment group. Data represent mean ± SEM. Each treatment group was normalized to the appropriate WT, uninjected control group and results expressed as percentage of WT mean. One-way ANOVA and *t*-tests of significance were used when applicable as measures of significance (GraphPad Prism software).

Results

The zebrafish parkin orthologue is highly conserved

BLAST searching of the zebrafish genome identified a single gene homologous to human *parkin*, ENSDARG00000021555, on chromosome 13. This 1 455 base transcript encodes a peptide sequence of 458 amino acids, of which 62% are identical and 75% similar to the sequence of the human gene. Within the E3 ubiquitin ligase domain (residues 1–76 in human parkin protein sequence), the human and zebrafish amino acid sequences display 61% identity and 78% similarity, respectively. In the functionally relevant RING domain regions (residues 238–294 and 418–450), the amino acid identity rises to 75–78% identity and 89–93% similarity. We confirmed the *in silico* results by amplifying reverse-transcribed zebrafish cDNA using *parkin*-specific primers and then sequencing the PCR products. Overlapping sequences were aligned and the consensus compared to *in silico* annotation. The genomic structure of zebrafish *parkin* is identical to the human gene, with a total of 12 translated exons. The sequence data we obtained from RT-PCR largely confirmed the annotated Ensembl sequence, with the exception of several single nucleotide polymorphisms (SNPs). Most of these single nucleotide polymorphisms were synonymous at the amino acid level (SNPs at positions 12, 27, 63, 69, 162, 210, 787, 1119, 1324 if the A in the ATG is +1). The only discrepancy between the *in silico* data and our own sequencing data resulting in a change of the protein sequence was a G substitution at position +112 in the highly conserved E3 ubiquitin ligase (UBL) domain, resulting in an alanine residue at codon 38. In contrast, the published Ensembl sequence at this position is a T, which would encode a serine. Of note, the corresponding residue in the human Parkin protein sequence is also an alanine.

Parkin is expressed throughout zebrafish embryonic development and in adult tissues

We used RT–PCR to characterize the expression pattern of *parkin* in zebrafish embryos at various stages and in different adult tissues. *parkin* mRNA was detectable throughout embryogenesis, from ~5 hpf until 5 dpf (Fig. 1A). Similarly, *parkin* is expressed ubiquitously in adult zebrafish, with variable levels of transcript detectable in all organs tested (Fig. 1B).

MO targeting of exon splice sites partially inactivates parkin in zebrafish embryos

Injection of the MOs e9i9 and i9e10 (see Materials and Methods section) resulted in complete (1 dpf) or partial (3 dpf) abolishment of full-length WT PCR product (Fig. 1C). Sequencing of the smaller band on the PCR gel revealed the primary product was indeed missing MO-targeted exon 9 from the mRNA, which results in deletion of 51 amino acids from the in-between ring (IBR) domain of Parkin. The amount of this product was greatly reduced in the knockdown embryos as compared to the WT, uninjected mRNA extracts, suggesting that nonsense-mediated decay may also affect the amount of mature *parkin* mRNA in MO-injected embryos.

Complex I activity is reduced in parkin knockdown embryos

We next measured mitochondrial respiratory chain complex I activity to determine whether this abnormality of energy metabolism, currently considered to be of crucial relevance in the pathogenesis of human *parkin*-linked Parkinson's disease,

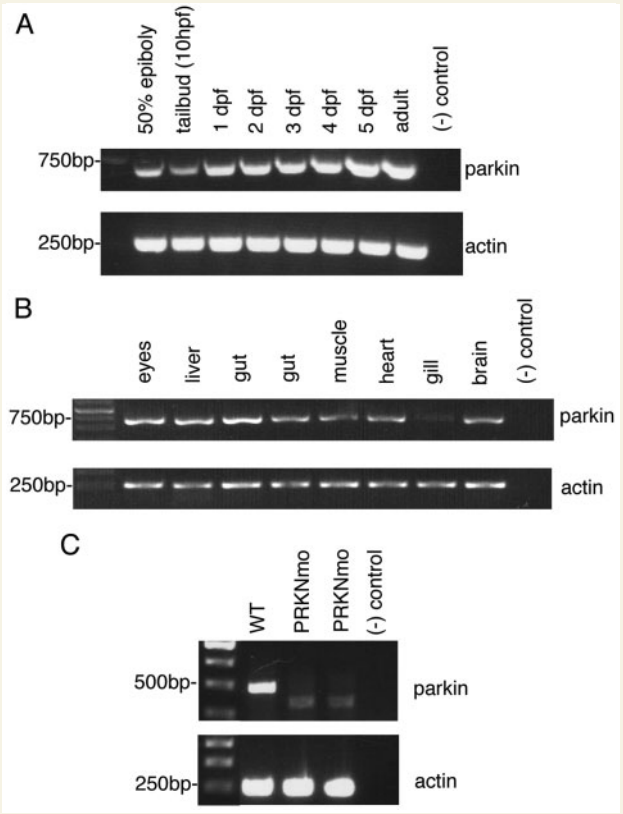

Figure 1 *parkin* mRNA expression in developing embryos, adult tissues and *parkin* knockdown embryos. (**A**) RT–PCR from cDNA reverse transcribed from total embryo mRNA extracted at various time points, illustrating *parkin* expression at early developmental stages. (**B**) RT–PCR from cDNA reverse transcribed from total mRNA in various adult zebrafish tissues, indicating that *parkin* is ubiquitously expressed. (**C**) RT–PCR from WT (uninjected) and MO-injected embryos, at 3 dpf. RT–PCR for *β-actin* is shown as a positive control; the negative control is a sample lacking mRNA in reverse transcription.

is also manifest in *parkin* knockdown zebrafish embryos. Spectrophotometric assessment of complex I activity was lower by an average of 45% in *parkin* knockdown zebrafish compared to either WT embryos or control MO embryos (Fig. 2; $P<0.05$). In contrast, there was no significant difference in complex II activity in *parkin* knockdown embryos relative to both WT and control MO embryos (mean ± SD, WT 0.26 ± 0.1, control MO 0.31 ± 0.09, *parkin* MO 0.19 ± 0.03). Likewise measurement of complexes III and IV showed no significant difference in activity of either complex in the parkin knockdown embryos relative to both WT and control MO embryos, although there was a trend towards a reduction in complex IV activity (mean ± SD, Complex III: WT 85.75 ± 19.7, control MO 67.62 ± 16.4, *parkin* MO 74.7 ± 5.7, Complex IV: WT 9.32 ± 3.7, control MO 9.55 ± 3.6, *parkin* MO 5.87 ± 1.5). These findings are thus in good agreement with the current view that it is complex I which is specifically affected in *parkin*-related Parkinson's disease as well as in the more common, sporadic form of Parkinson's disease.

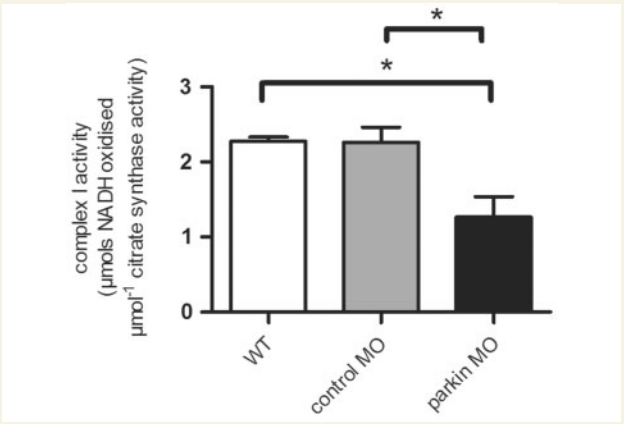

Figure 2 *parkin* knockdown results in specific mitochondrial complex I deficiency. Mitochondrial complex I activity, measured relative to the mitochondrial marker enzyme citrate synthase, in extracts of WT, control MO and *parkin* MO-injected embryos at 3 dpf. Complex I activity in *parkin* MO embryo extracts is significantly reduced (*$P<0.05$).

Knockdown of zebrafish parkin results in dopaminergic neuronal cell loss and increased susceptibility to MPP+

Using *in situ* hybridization with an antisense RNA probe for TH, we investigated the effect of *parkin* knockdown on the number of diencephalic dopaminergic neurons. Dopaminergic neurons ascending to the striatum (populations 1, 2, 4 and 5) according to terminology developed by Rink and Wullimann (2002a) were counted in WT uninjected embryos, control MO-injected embryos and *parkin* MO-injected embryos at 3 dpf. We calculated the mean number of these diencephalic dopaminergic neurons for WT embryos in each of three experiments and used this mean as the standard number of dopaminergic neurons, which was set to 100% in order to normalize the data. The number of ascending dopaminergic neurons present in each treatment group was then calculated as the percentage of the number of these dopaminergic neurons in WT zebrafish. *parkin* knockdown embryos showed a significant reduction in the number of diencephalic dopaminergic neurons by ~20% ($P<0.01$) already at 3 dpf. In contrast, the number of dopaminergic neurons in control MO-injected embryos did not differ significantly from WT uninjected embryos (Fig. 3A).

Knockdown of zebrafish parkin results in increased susceptibility to MPP+

We next determined whether *parkin* knockdown results in an increased susceptibility to the mitochondrial neurotoxin MPP+. Treatment of *parkin* knockdown zebrafish embryos with MPP+ resulted in a significantly greater reduction in the number of diencephalic dopaminergic ascending neurons by ~50% ($P<0.001$), suggesting a synergistic effect of *parkin* knockdown and MPP+ treatment (Fig. 3B). In contrast, MPP+ exposure of

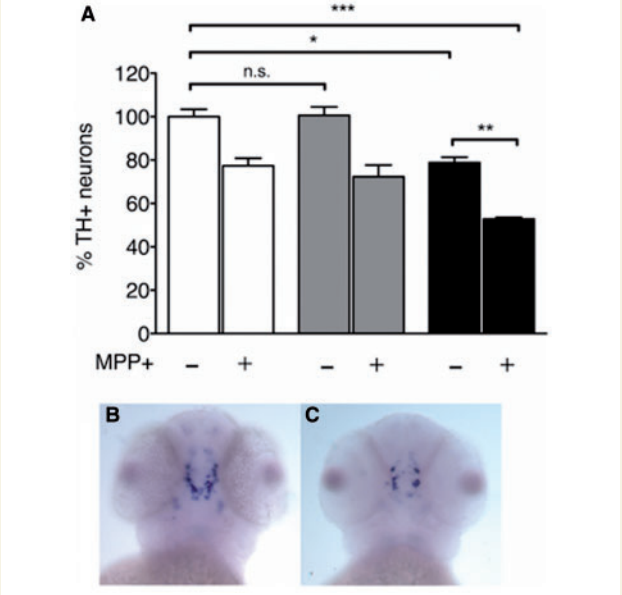

Figure 3 Knockdown of *parkin* via MO injection decreases diencephalic dopaminergic neuron count. (**A**) Diencephalic dopaminergic neuron percentages counted by *in situ* hybridization using TH probe in WT (white bars), control MO-injected embryos (gray bars) and *parkin* MO-injected embryos (black bars) at 3 dpf. Concomitant treatment with MPP+ (indicated on *x*-axis) results in further decrease in dopaminergic neurons (*$P<0.05$; **$P<0.01$, ***$P<0.001$, one-way ANOVA). Error bars represent the SEM. (**B**) *In situ* hybridization with TH probe (blue staining) as a marker for dopaminergic neurons in 3 dpf zebrafish embryo injected with control MO. (**C**) Markedly reduced TH staining in 3 dpf zebrafish embryos injected with *parkin* MO and treated with MPP+.

WT and control MO-injected zebrafish embryos only resulted in a reduction by ~25% ($P<0.01$).

The effect of parkin knockdown on dopaminergic neurons is specific

We next undertook double-staining with a TH antibody and an *in situ* probe for *aromatic amino acid decarboxylase (AADC)*. This allowed us to discriminate between catecholaminergic and serotonergic neurons, respectively. While both neuronal subpopulations express *AADC* (red in Fig. 4), only catecholaminergic neurons express *TH* (green in Fig. 4) as well. Co-expression of both markers (yellow in Fig. 4) is thus specific for catecholaminergic neurons such as the ascending diencephalic dopaminergic neurons (Fig. 4A). These co-expression studies confirmed that the marked reduction of diencephalic neurons in *parkin* knockdown zebrafish embryos was specific for these dopaminergic neurons and further supported our assumption that the *TH in situ* based cell counting results were not simply reflecting down-regulation of *TH* transcription. In contrast, the (red) serotonergic neurons of the Raphe nuclei appeared similar in WT and *parkin* knockdown zebrafish embryos.

To exclude the possibility that the effect we observed was due to generalized impairment of brain development (either due to a specific effect of *parkin* knockdown or due to a non-specific toxic effect of the MO injections) rather than being specific to dopaminergic neurons we next undertook staining with anti-HuC/D antibody, an early pan-neuronal marker for post-mitotic neurons (Park *et al.*, 2000). The HuC staining resulted in a similar staining pattern and intensity in WT uninjected and *parkin* knockdown embryos at 30-hpf (Fig. 4B). To assess further the specificity of the *parkin* knockdown effect we also undertook staining of spinal motor neurons, using anti-Islet 1 antibodies. The staining pattern was identical in WT uninjected and *parkin* knockdown embryos at 30 hpf (Fig. 4C). Overall, our morphological studies suggest that *parkin* knockdown has a largely selective effect on the number of ascending dopaminergic neurons rather than leading to gross morphological changes or widespread neuronal cell loss within the zebrafish brain.

Parkin deficiency does not alter larval swimming behaviour

Because of the locomotor defects observed in Parkinson's disease patients, we next tested whether *parkin* knockdown results in changed swimming behaviour of 5 dpf larvae. We compared spontaneous swimming behaviour at 5 dpf in WT uninjected, control MO-injected, and *parkin* MO-injected larvae. The mean distance moved over three experiments for WT, control MO and *parkin* MO-injected larvae did not differ significantly (Fig. 5), suggesting no obvious differences in swimming ability between groups. We noted a trend towards reduced total distance moved in both control MO and *parkin* MO-injected larvae, but this result was not significant ($P>0.05$).

Electron microscopy studies reveal morphologically normal mitochondria, but electron-dense material in the t-tubules in *parkin* knockdown zebrafish

Drosophila parkin mutants develop apoptotic muscle necrosis and swollen mitochondria with broken cristae (Greene *et al.*, 2003). In contrast, both muscle tissue as such and the mitochondria in particular are morphologically normal in *parkin* knockout mice (Palacino *et al.*, 2004). We therefore undertook TEM of fast muscle cells in the somites of the zebrafish trunk to determine whether abnormalities similar to those found in *Drosophila* are manifest in the absence of Parkin function. TEM did not reveal any obvious morphological abnormalities of the mitochondria in *parkin* knockdown zebrafish embryos. In particular, the mitochondria were not swollen and the cristae remained intact. Furthermore, there was no evidence for apoptotic muscle necrosis (Fig. 6A–D). Surprisingly, however, electron-dense material was observed in the t-tubules of the *parkin* MO-injected zebrafish (Fig. 7A–F). These invaginating tubules allow for fast depolarization of the interior of the cell and may thus play a role in Ca^{2+} mediated muscle contraction. In initial swimming tests *parkin* MO-injected larvae did not display obvious locomotive defects

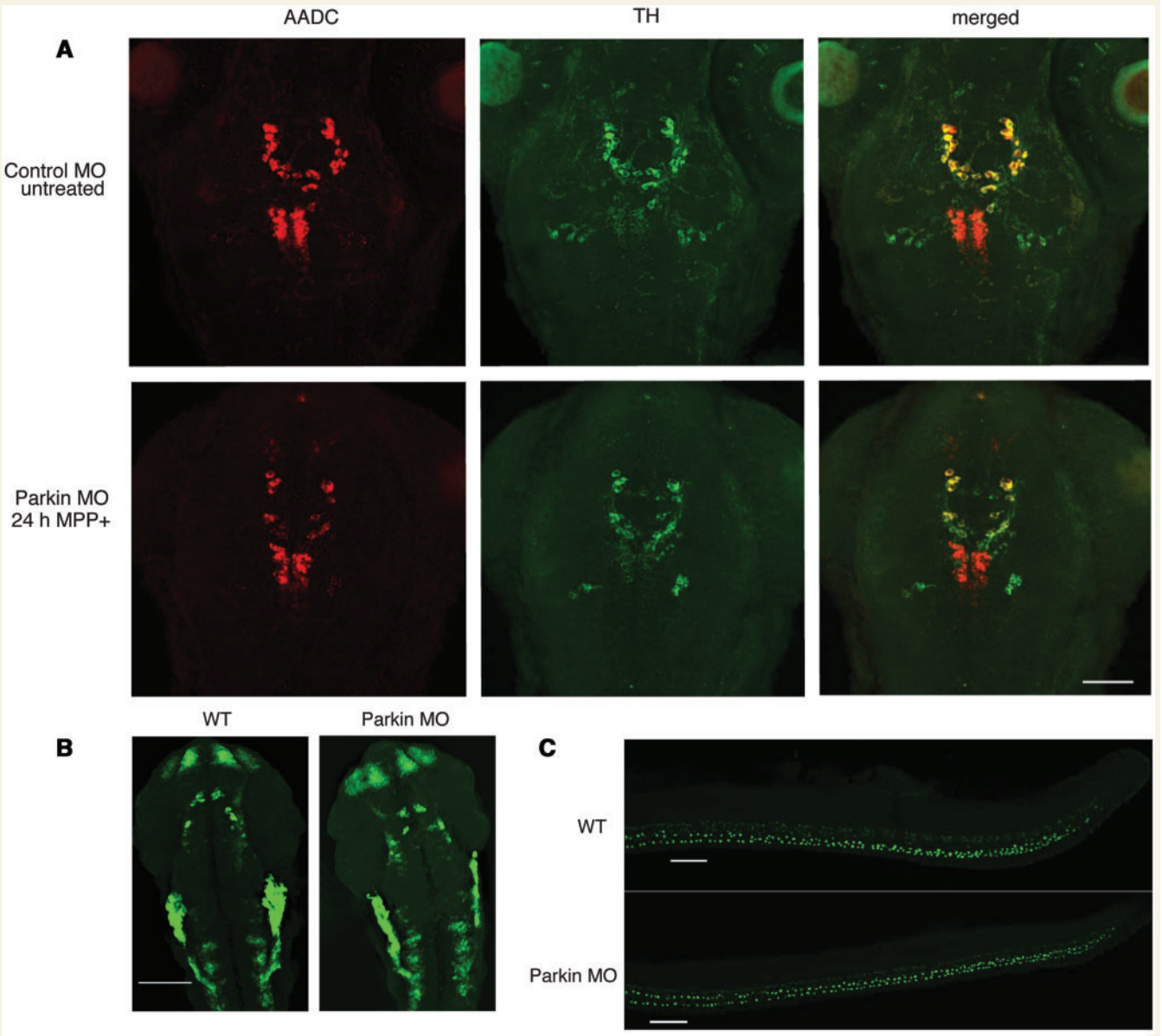

Figure 4 Knockdown of *parkin* via MO injection is specific to dopaminergic neurons. (**A**) Whole-mount *in situ* hybridization for *AADC* (red) combined with immunohistochemical labelling using anti-TH antibody (green). Serotonergic neurons (red staining) are preserved in WT, untreated embryos and *parkin* knockdown embryos exposed to MPP+. Neurons positive for both *AADC* and TH (yellow staining) are reduced in *parkin* knockdown embryos treated with MPP+, indicating specific loss of dopaminergic cells in the posterior tuberculum. (**B**) Immunostaining with anti-HuC/D antibody labelling post-mitotic neurons shows similar morphology in 30 hpf WT and *parkin* knockdown embryos. (**C**) Immunostaining with anti-Islet-1 antibody indicates normal spinal cord motor neuron patterning in both 30 hpf WT and *parkin* knockdown embryos.

(see above). As the temporal activity of the MO is limited though this t-tubule alterations may be tolerated at larval stages but could become more severe in mutants with permanent Parkin depletion.

Discussion

We have developed a new vertebrate model to study the biochemical and morphological consequences of Parkin deficiency using MO-mediated knockdown of the zebrafish orthologue of the *parkin* gene. Comparison with embryos injected with a mismatch MO give us high confidence that the defects we observe are attributable specifically to the loss of Parkin activity rather than being caused by non-specific off target effects of the MO antisense oligonucleotide.

parkin knockdown zebrafish embryos share a lower activity of the mitochondrial respiratory chain complex I with *parkin*-mutant human patients (Muftuoglu *et al*., 2004; Mortiboys *et al*., 2008). Mitochondrial impairment is currently considered to be of crucial

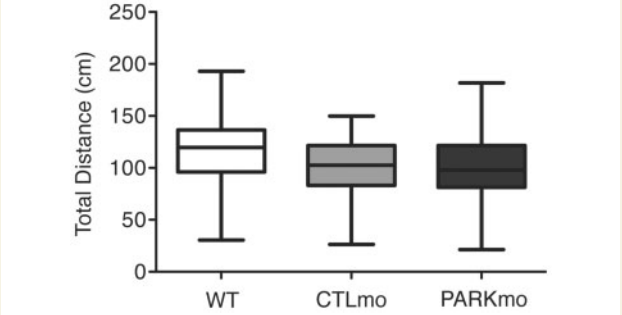

Figure 5 Swimming behaviour is not altered by *parkin* knockdown in 5 dpf larvae. Larvae placed in 4.5 ml of E3 embryo media per well in a 12-well plate were monitored for 5 min. The swimming larvae were tracked and analysed for total distance moved. No significant difference was observed between WT, control MO or *parkin* MO-injected larvae. Data shown represent three experiments.

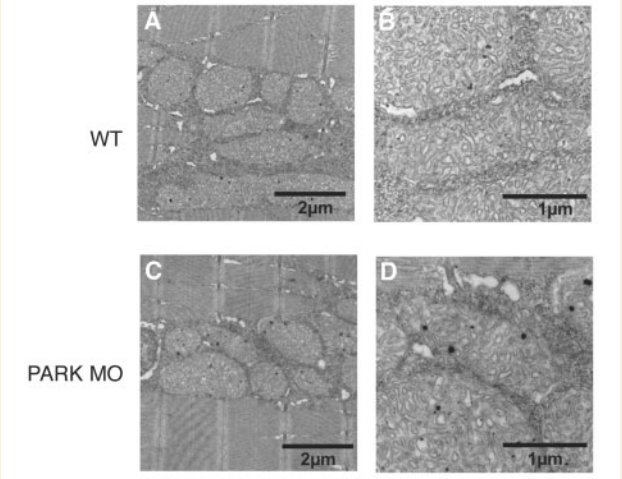

Figure 6 Gross mitochondrial morphology is similar in WT and *parkin* knockdown embryos. Longitudinal TEM sections of fast muscle cells in the trunk somites of 5 dpf WT zebrafish larva (**A** and **B**) and larvae injected with *parkin* MO (**C** and **D**). The overview (A and C, 10 000×) shows normal mitochondria and myofibrils in both WT and *parkin* knockdown zebrafish embryos. (B and D) Higher magnification (25 000, B and D) reveals identical shape and structure of the mitochondrial cristae in WT and *parkin* knockdown zebrafish.

importance in the pathogenesis not only of *parkin*-related Parkinson's disease, but also in sporadic Parkinson's disease (Schapira, 2008). Furthermore, there is some evidence that Parkin itself may also be involved in the pathogenesis of sporadic Parkinson's disease: Parkin is clearly present in Lewy bodies and its transcription is upregulated in the substantia nigra of patients with sporadic Parkinson's disease (Schlossmacher *et al*., 2002; Moran *et al*., 2007). Parkin protects against the toxicity associated with mutant alpha-synuclein *in vitro* and *in vivo* (Lo Bianco *et al*., 2002; Petrucelli *et al*., 2002).

Post-mortem tissue of *parkin*-mutant patients has not as yet been assessed for morphological abnormalities of the mitochondria (Cookson *et al*., 2008). The normal shape and integrity of mitochondrial cristae in the *parkin* knockdown zebrafish embryos that manifest impaired mitochondrial function could indicate that the latter leads to impaired mitochondrial morphology, but further studies are needed to clarify this issue. The MO antisense approach only causes transient loss of Parkin function. Other strategies such as targeted gene mutagenesis using zinc finger nucleases could be used to develop stable *parkin* knockout zebrafish lines which would then permit serial assessment of mitochondrial morphology at different ages (Woods and Schier, 2008). Similarly, t-tubule morphology and function could be assessed at different ages.

Morphological analysis revealed selective loss of dopaminergic neurons in *parkin* knockdown zebrafish embryos, a finding that contrasts both with the more widespread defects observed in *parkin* knockout *Drosophila*, and also with the absence of any robust morphological changes in *parkin* knockout mice (Greene *et al*., 2003; Perez and Palmiter, 2005). Such selective dopaminergic neuronal cell loss together with lower complex I activity mirrors the human disease better than any other currently available animal model for *parkin* disease. The future development of stable *parkin* knockout zebrafish lines (see above) will help to determine whether the observed loss of dopaminergic neurons is progressive. The increased susceptibility to MPP+ observed in our *parkin* knockdown zebrafish model is in keeping with data from *in vitro* studies, but contrasts with the lack of increased susceptibility to this neurotoxin in *parkin* knockout mice (Hyun *et al*., 2002; Thomas *et al*., 2007). We had previously demonstrated increased susceptibility to a different mitochondrial toxin, rotenone, in *parkin*-mutant patient tissue (Mortiboys *et al*., 2008).

In *Drosophila*, Parkin and PINK1 appear to act in the same mitochondrial pathway, resulting in the same phenotype when either (or both) of these genes is mutated (Clark *et al*., 2006; Park *et al*., 2006; Yang *et al*., 2006). *PINK1* knockdown in zebrafish results in a severe developmental phenotype (Anichtchik *et al*., 2008). In contrast, our morphological studies, in particular the normal staining pattern with the pan-neuronal marker HuC and the motor-neuron specific marker Islet-1, suggest a considerably more selective effect of *parkin* knockdown than *PINK1* knockdown. The MOs used in our study resulted in a 51 amino acid deletion within the in-between ring domain of *parkin*. The in-between ring domain augments binding of E2 proteins and the subsequent ubiquination of proteins such as synphilin-1 (Beasley *et al*., 2007). The resulting partially deleted Parkin protein in our study may thus have decreased enzymatic activity as a ubiquitin 3 ligase, but could nevertheless have some remaining functional activity since other crucial parts of the protein such as the ubiquitin ligase (UBL) domain and the two RING domains remained intact. In contrast, MOs directed against the ATG translation initiation site were used in the zebrafish *PINK1* knockdown studies (Anichtchik *et al*., 2008). This might have led to a more complete suppression of PINK1 function. It is nevertheless difficult to understand why PINK1 deficiency results in such marked impairment of gross brain development in zebrafish embryos,

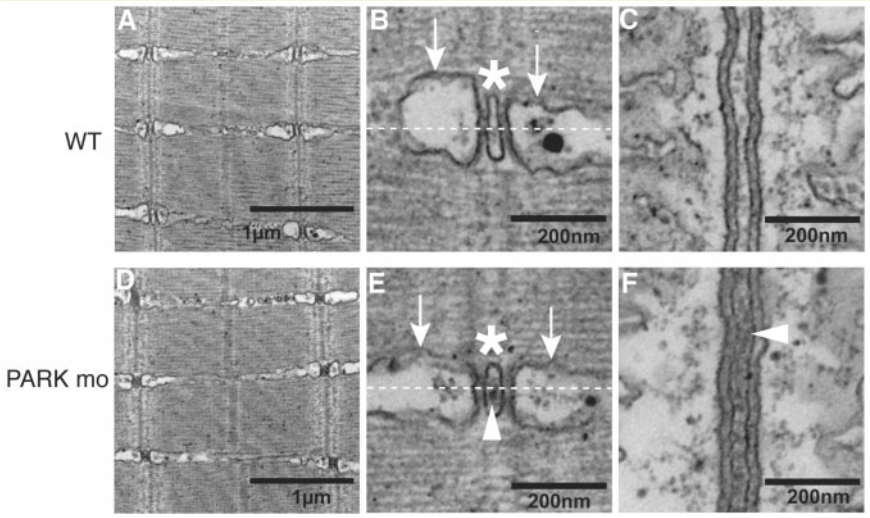

Figure 7 Electron-dense staining in t-tubules of *parkin* knockdown embryos. Longitudinal TEM sections of fast muscle cells in the trunk somites of 5 dpf WT zebrafish larva (**A–C**) and larvae injected with *parkin* MO (**D–F**). The increase in electron dense material within the t-tubules in the *parkin* knockdown zebrafish embryos is already visible at lower magnification (25 000× A and D). At higher magnification (60 000×) the triad, formed by the t-tubule (C and F, asterisk) and the terminal cisternae of the sarcoplasmic reticulum (SR, C and F, arrow) becomes more clearly visible and the increase in electron dense material thus more apparent. The dashed line depicts the plane of the sections shown in (B and E). In the *parkin* knockdown larvae the t-tubules contain electron dense material (E an F, arrowhead), the SR and the myofibrils appear normal.

but does not result in any morphological abnormalities in *PINK1* knockout mice (Zhou *et al*., 2007; Gautier *et al*., 2008). Post-mortem studies have not as yet been undertaken in human patients with *PINK1* mutations, but the clinical phenotype is obviously only suggestive of regionally selective neuronal cell loss rather than gross brain dysmorphology. Both the *PINK1*- and our *parkin* knockdown zebrafish studies nevertheless suggest that the encoded proteins may have a crucial role for the development and/or survival of neurons at early developmental stages. This may appear counter-intuitive for an adult-onset neurodegenerative condition, but is in keeping with recent observations describing abnormalities in key developmental mechanisms, namely microRNA signalling, in late-onset sporadic Parkinson's disease (Kim *et al*., 2007).

parkin knockdown zebrafish embryos did not display an overt behavioural phenotype. It is currently thought that human patients have to lose at least two thirds of their dopaminergic neurons in the substantia nigra before they develop any motor symptoms of Parkinson's disease. The loss of dopaminergic neurons observed in our model system (~25%) may therefore not have been sufficient enough to result in a motor phenotype. Complete and longer lasting loss of Parkin function in stable *parkin* knockout lines will reveal whether dopaminergic neuron loss becomes more pronounced both during embryonic development and at later stages which may in turn lead to a more marked behavioural phenotype.

The observation of electron-dense material in the t-tubules of the *parkin* knockdown zebrafish embryos was a serendipitous finding. T-tubules are rich in L-type Ca^{2+} channels (Brette and Orchard, 2007). Dopaminergic neurons in the substantia nigra pars compacta (SNpc) rely on L-type Ca^{2+} channels for pacemaking with a much larger Ca^{2+} influx in these neurons than in other cells (Surmeier, 2007). This increased Ca^{2+} load of the dopaminergic neurons could in turn lead to a considerably higher energy demand to transport Ca^{2+} back out of the cells, consuming ATP produced by oxidative phosphorylation in the mitochondria. Future studies are needed to determine whether the observed impairment of mitochondrial function in *parkin*-linked Parkinson's disease contributes to neuronal cell death not only due to its effect on overall energy production, but also because of the important role of mitochondria in the maintenance of intracellular Ca^{2+} homeostasis (Szabadkai and Duchen, 2008).

Zebrafish embryos are highly amenable to high throughput drug screening and might therefore be a useful new tool to work towards the identification of disease-modifying therapy for Parkinson's disease. We have already provided proof of principle data in our *DJ-1* knockdown studies that zebrafish embryos can be a useful tool to investigate compounds for their putative neuroprotective effect (Bretaud *et al*., 2007). Similarly, we will now be able to use *parkin* knockdown zebrafish embryos to rapidly assess promising substances *in vivo* in this new vertebrate animal model of Parkin deficiency. The recent development of an enhancer trap transgenic zebrafish line, ETvmat2:GFP, with green fluorescent monoaminergic neurons will further facilitate the rapid assessment of drugs for a putative neuroprotective effect on dopaminergic neurons in high throughput compound screens (Wen *et al*., 2008). It has been suggested that small molecules should be tested for their ability to induce *parkin* transcription to identify disease-modifying treatment for Parkinson's disease (Butcher, 2005). Genetically modified zebrafish embryos may also be an ideal tool to undertake such studies.

Acknowledgements

We would like to acknowledge the contribution of Dr S. Bretaud to the early stages of this project. We would also like to thank the aquarium staff at CDBG for zebrafish maintenance and Luise Jennen for excellent help with TEM recording.

Funding

Parkinson's Disease Society (PDS, G-0608); Research into Ageing (RiA, 282 to O.B. and P.W.I.); Medical Research Council (MRC Centre Grant to P.W.I.); Bundesministerium fuer Bildung und Forschung (BMBF, BioFuture Grant 0311889 to K.V. and R.W.K.); Wellcome Trust (GR077544AIA) for the University of Sheffield Light Microscopy Facility.

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