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The cellular and molecular progression of mitochondrial dysfunction induced by 2,4-dinitrophenol in developing zebrafish embryos

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Abstract

The etiology of mitochondrial disease is poorly understood. Furthermore, treatment options are limited, and diagnostic methods often lack the sensitivity to detect disease in its early stages. Disrupted oxidative phosphorylation (OXPHOS) that inhibits ATP production is a common phenotype of mitochondrial disorders that can be induced in zebrafish by exposure to 2,4-dinitrophenol (DNP), a FDA-banned weight-loss agent and EPA-regulated environmental toxicant, traditionally used in research labs as an uncoupler of OXPHOS. Despite the DNP-induced OXPHOS inhibition we observed using *in vivo* respirometry, the development of the DNP-treated and control zebrafish were largely similar during the first half of embryogenesis. During this period, DNP-treated embryos induced gene expression of mitochondrial and nuclear genes that stimulated the production of new mitochondria and increased glycolysis to yield normal levels of ATP. DNP-treated embryos were incapable of sustaining this mitochondrial biogenic response past mid-embryogenesis, as shown by significantly lowered ATP production and ATP levels, decreased gene expression, and the onset of developmental defects. Examining neural tissues commonly affected by mitochondrial disease, we found that DNP exposure also inhibited motor neuron axon arbor outgrowth and the proper formation of the retina. We observed and quantified the molecular and physiological progression of mitochondrial dysfunction during development with this new model of OXPHOS dysfunction, which has great potential for use in diagnostics and therapies for mitochondrial disease.

Keywords

Zebrafish; Embryo; Energy metabolism; Motor neuron; Retina; *In vivo* respirometry

1. Introduction

Most eukaryotic cells are dependent upon mitochondrial energy metabolism through oxidative phosphorylation (OXPHOS) for the majority of their ATP synthesis. Single cells

may contain hundreds of mitochondria and each organelle is equipped with at least one (but typically more) copies of the mitochondrial genome, which encodes only 13 of the hundreds of genes required for OXPHOS (Chinnery and Hudson, 2013). Furthermore, patients carrying identical mutations may present with varied pathologies affecting different organ systems (Haas et al., 2008). These complex features of mitochondrial biology have complicated the development of reliable diagnostics and treatment strategies for mitochondrial disease, especially pediatric disease (Koenig, 2008). As a result, many questions remain as to how mitochondrial dys-function progresses during development. Mitochondrial dysfunction often occurs in tissues with high energetic needs, and a large proportion of patients develop nervous system pathologies that result in sensory and motor deficits (DiMauro and Schon, 2008; Koenig, 2008). The sensitivity of the nervous system may be due to the high energy demands required to maintain the ion gradients across neuronal membranes and neurons have a low capacity to store ATP for later use (Erecinska et al., 2004). Post-mitotic neuronal cells also accumulate deleterious mitochondrial DNA (mtDNA) mutations at a high rate, which can affect expression of proteins involved in OXPHOS and exacerbate mitochondrial dysfunction (Bender et al., 2006; Zsurka and Kunz, 2013). In addition to their critical role as ATP generators, mitochondria also control calcium homeostasis, generate reactive oxygen species (ROS), and regulate apoptosis and many other cellular signaling cascades. These non-ATP generating processes are thought to play an underappreciated role in the development of tissue-specific pathologies that result from mitochondrial disease (Raimundo, 2014), and may in part explain why metabolically-demanding tissues (i.e., heart and central nervous system) are not equally affected by mitochondrial dysfunction.

It remains unclear how mitochondrial dysfunction produces the severe sensory and motor deficits seen in patients (DiMauro and Schon, 2008). Hindering the development of therapeutics is the dearth of experimental systems that model progressive mitochondrial disease and mitochondrial dysfunction in vivo. Here we have disrupted the mitochondrial proton gradient in developing zebrafish embryos with 2,4-dinitrophenol (DNP), a FDA-banned weight loss agent and EPA-regulated environmental toxicant, which is used experimentally as an uncoupler of OXPHOS (Harris and Corcoran, 1995). DNP transports protons across the mitochondrial inner membrane, altering the proton gradient and inhibiting ATP production via OXPHOS (Lou et al., 2007). ROS are a byproduct of OXPHOS and as DNP decreases OXPHOS via dissipation of the proton gradient, DNP treatment will decrease ROS production. Indeed, work in mice has demonstrated that DNP treatment results in lowered ROS production (Caldeira da Silva et al., 2008), an observation that follows other work postulating that mitochondrial uncoupling is an actively regulated process employed to decrease oxidative damage and stress (Brand, 2000). In addition to the detrimental effects of ROS, recent work has shown that mitochondrially-generated ROS are critical signaling molecules that regulate a growing list of cell functions (Raimundo, 2014), including regulation of the cell cycle (Trachootham et al., 2008).

In healthy cells, mitochondria are plastic and cells respond to changing energy demands by regulating ATP synthesis, as well as altering the number of mitochondria (Liesa and Shirihai, 2013). We were interested in quantifying the effects of DNP-induced mitochondrial dysfunction on the molecular and cellular development of embryos, in order to determine

the extent to which embryos are capable of mounting molecular defenses to compensate for chronic mitochondrial dysfunction. Within hours of exposure to low levels of DNP, zebrafish embryos showed significant changes in respiration, ATP levels, ROS output, and gene expression of key mitochondrial proteins. Our quantitative RT-PCR (qPCR) results revealed that embryos induce mitochondrial bio-genesis soon after exposure to DNP. However, this was not sustained. The developmental consequences of continued mitochondrial dysfunction became significant during the second half of embryogenesis. During this period, measures of mitochondrial biogenesis decreased, and the development of the nervous system, a major target of mitochondrial disease, was inhibited. Using quantitative analysis of the development of the retina and primary motor neuron development, we found that continued DNP exposure inhibited retinal laminae formation and axon outgrowth. Together, these results provide insights into the progression of congenital mitochondrial disease, revealing the developmental consequences of sustained mitochondrial dysfunction.

2. Results

2.1. DNP exposure impairs mitochondrial function in zebrafish embryos

We were interested in examining the effects of chronic DNP exposure on zebrafish metabolism during embryonic development, given that DNP acts to disrupt the proton gradient responsible for driving ATP synthesis in mitochondria. The *in vivo* respiration of individual developing zebrafish embryos was quantified using techniques we developed that measure oxygen consumption rates (OCR) under basal conditions and in the presence of specific drugs used to inhibit or maximize respiration (Stackley et al., 2011). Beginning at 3 h post fertilization (hpf), at the ~1000 cell blastomere stage, embryos were treated with 0.5 μ M DNP, a concentration that is approximately one third lower than previously established LC_{10} levels for zebrafish (Weil et al., 2009) and one tenth the EPA-recommended levels for bodies of water where fish may be caught for consumption (Harris and Corcoran, 1995). 3 h after exposure to DNP, the basal OCR from DNP-treated embryos was 44.0 ± 2.1 pmol O_2 /min ($n = 8$) compared to the 29.2 ± 2.1 pmol O_2 /min average control level ($n = 9$; $p = 0.003$), indicating that this low dose of DNP had disrupted the mitochondrial proton gradient, increasing respiration (Fig. 1A).

The DNP-induced increase in oxygen consumption was not maintained over the course of embryonic development. At 24 hpf, the average basal respiration rate in embryos chronically treated with DNP was 55.2 ± 2.5 pmol O_2 /min ($n = 38$), and not significantly different ($p=0.42$) from the OCR of control animals which had increased to 53.1 ± 2.4 pmol O_2 /min ($n=36$; Fig. 1B). By 48 hpf, the rate of basal respiration increased significantly in the control embryos to 82.1 ± 3.9 pmol O_2 /min ($n=38$; $p<0.0001$), and increasing again at 72 hpf, reaching 96.0 ± 4.6 pmol O_2 /min ($n=37$; $p=0.02$). At 50.0 ± 2.9 pmol O_2 /min ($n=37$), the basal respiration rate of 48 hpf DNP-treated embryos did not increase from the 24 hpf time point ($p=0.84$), and was significantly lower than the control rate ($p<0.0001$). At 72 hpf, the average basal respiration of DNP-treated embryos was just 55.3 ± 2.7 pmol O_2 /min ($n=37$), which was not different from their 48 hpf level ($p=0.15$), and was significantly less than the basal rates of control embryos ($p<0.0001$). Together these data reveal that although DNP

induced the expected increase in cellular respiration rates in the initial hours after treatment, this increased basal respiration was not maintained. In addition, DNP exposure prevented the normal increase in basal respiratory output over time, as this measure stayed significantly below control levels over the subsequent 3 days of embryonic development.

Using specific compounds to alter mitochondrial physiology, we performed a more detailed mechanistic dissection of mitochondrial respiration at 24, 48 and 72 hpf. We recorded basal respiration rates from all of the embryos tested (Fig. 1B) and subsets of these embryos were randomly selected to receive the specific compounds used to interrogate specific aspects of mitochondrial respiration. In vivo respiration was recorded from intact embryos before and after the addition of carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) at a level we have shown to fully dissipate the electrochemical gradient and induce maximal respiration as mitochondria attempt to maintain their membrane potential OXPHOS (Stackley et al., 2011). At 24 hpf, the basal respiration rate from the control animals before the addition of FCCP was 40.3 ± 2.8 pmol O₂/min ($n=14$). After exposing the embryos to FCCP, the average respiration rate of the control animals increased significantly by 9.8 ± 1.3 pmol O₂/min compared to their basal rates ($n=14$; $p<0.0001$), demonstrating that the applied concentration of FCCP was sufficient to push control embryos to their maximal respiration level which at this early time point is quite close to their unperturbed basal respiration rate (Stackley et al., 2011). Before addition of FCCP, the basal respiration of the DNP-treated animals was 47.6 ± 5.5 pmol O₂/min, but FCCP treatment did not significantly increase respiration rates when compared to the basal respiration of that subset of animals (4.12 ± 2.83 pmol O₂/min average increase, $n=12$; $p=0.11$). In addition, the FCCP-induced maximal respiration rates at 24 hpf were not significantly different between control (51.3 ± 3.6 pmol O₂/min, $n=16$) and DNP-treated embryos (51.7 ± 5.6 pmol O₂/min, $n=12$; $p=1.0$; Fig. 1C). At 48 hpf, the average maximal respiratory rate of control animals increased significantly to 92.7 ± 11.4 pmol O₂/min ($n=11$; $p=0.002$; Fig. 1C), an average increase of 21.9 ± 9.5 pmol O₂/min over basal rates ($p=0.04$). At 48 hpf, FCCP exposure increased respiration of the DNP-treated embryos 4.9 ± 4.9 pmol O₂/min over basal levels ($n=9$, $p=0.73$). Reaching only 47.1 ± 8.0 pmol O₂/min at 48 hpf (Fig. 1C), the maximal respiration rate of DNP-treated animals did not increase between 24 and 48 hpf ($p=0.50$). At 72 hpf, FCCP exposure did not significantly increase respiration rates of DNP treated embryos, inducing only a 16.7 ± 7.0 pmol O₂/min increase in respiration ($n=8$; $p=0.29$), compared to the 22.7 ± 7.5 pmol O₂/min increase in the respiration of 72 hpf control fish ($n=10$; $p=0.01$). The maximal respiration rate of the 72 hpf control embryos reached 106.9 ± 6.6 pmol O₂/min (Fig. 1C), which was not significantly greater than the maximal rate measured at 48 hpf ($p=0.22$). At 71.0 ± 6.7 pmol O₂/min, the FCCP-induced maximal respiration rate of the 72 hpf DNP-treated embryos showed a significant increase over 48 hpf levels ($n=8$; $p=0.02$), but did not reach control levels ($p<0.001$; Fig. 1C). Overall, these results show that unlike control animals, the DNP-treated embryos did not produce the expected FCCP-induced increase in respiration over basal respiration rates. In addition, the maximal respiration rates of the DNP-treated embryos were significantly less than the control group after 24 hpf. Together these data indicate that DNP-treated embryos respire near their maximal respiratory capacity and suggest that DNP exposure prevents zebrafish embryos from responding to their metabolic demands.

Oligomycin inhibits the mitochondrial ATP synthase, thus the level of oligomycin-sensitive respiration (the difference between the OCR after oligomycin treatment and the average basal OCR) indicates the amount of respiration that is linked to mitochondrial ATP production (ATP-linked respiration). After recording basal rates, embryos were exposed to oligomycin. At all time points, DNP-treated embryos had significantly decreased levels of ATP-linked respiration compared to control animals ($p=0.0018$ at 24 hpf, $p=0.0006$ at 48 hpf, and $p=0.0029$ at 72 hpf; Fig. 1D). At 24 hpf, the ATP-linked respiration of control animals was 46.3 ± 2.5 pmol O_2 /min ($n=11$), increasing significantly to 58.8 ± 3.1 pmol O_2 /min at 48 hpf ($n=9$; $p=0.0136$) and reaching 73.5 ± 12.6 pmol O_2 /min at 72 hpf ($n=7$; not significantly greater than the 48 hpf levels, $p=1.0$). In contrast, the ATP-linked respiration of DNP-treated embryos stalled at the 24 hpf level (32.2 ± 2.9 pmol O_2 /min, $n=15$); neither the ATP-linked OCR at 48 hpf (28.7 ± 3.2 pmol O_2 /min, $n=12$) nor 72 hpf (29.2 ± 4.3 pmol O_2 /min) showed significant daily changes ($p=0.91$ and $p=0.99$, respectively). These data indicate that chronic DNP exposure decreases the efficiency of mitochondrial ATP production compared to control animals.

After recording basal respiration rates, embryos were treated with sodium azide to inhibit mitochondrial respiration and ATP hydrolysis, revealing the small proportion of oxygen consumption that is contributed by non-mitochondrial oxidases (Brand and Nicholls, 2011). Non-mitochondrial respiration was not significantly different between 24 and 48 hpf for the control animals, but increased significantly between 48 and 72 hpf ($p=0.27$ between 24 and 48 hpf and $p=0.004$ between 48 and 72 hpf; Fig. 1E). At 24 hpf, non-mitochondrial OCR of control fish was 12.5 ± 3.9 pmol O_2 /min ($n=12$), increasing to 14.3 ± 1.5 pmol O_2 /min at 48 hpf ($n=18$) and 22.4 ± 1.7 pmol O_2 /min at 72 hpf ($n=19$). Tracking the daily levels of non-mitochondrial respiration in the DNP-treated embryos revealed a significant change between 24 and 48 hpf ($p=0.05$), but no increase between 48 and 72 hpf ($p=0.50$). With an OCR of 8.9 ± 0.9 pmol O_2 /min, the average DNP-treated embryos' non-mitochondrial respiration rate was significantly less than control levels at 24 hpf ($n=11$; $p=0.036$). At 48 hpf, the average non-mitochondrial respiration rate for DNP-treated embryos was 12.9 ± 1.4 pmol O_2 /min ($n=18$), which was not significantly different from control levels ($p=0.24$). At 72 hpf the azide-resistant oxidase activity of the DNP-treated embryos was 15.4 ± 2.2 pmol/min at ($n=19$), again significantly less than control levels ($p=0.015$).

These data indicate that absolute levels of non-mitochondrial oxygen consumption were decreased by DNP exposure, but when non-mitochondrial respiration is considered as a proportion of the total respiratory output of the embryos (non-mitochondrial respiration/basal respiration), the contribution of non-mitochondrial OCR was not different between the DNP-treated and control animals. At 24 hpf, the mean percent of respiration made up of the non-mitochondrial component was $21.5 \pm 2.3\%$ ($n=12$) for control animals, and $16.4 \pm 1.5\%$ ($n=11$) for DNP-treated embryos ($p=0.13$). At 48 hpf, the control animal percent was $18.8 \pm 2.1\%$ ($n=18$) and the DNP-treated embryos reached $25.2 \pm 4.3\%$ ($n=17$; $p=0.09$). At 72 hpf, we found that the non-mitochondrial percentage of the total OCR was $23.8 \pm 2.6\%$ ($n=19$) for control animals and $26.5 \pm 3.6\%$ for DNP-treated embryos ($p=0.98$). These data reveal that while the absolute levels of non-mitochondrial oxidase activity of DNP-exposed embryos were attenuated throughout most of embryogenesis, DNP exposure did not

significantly alter the non-mitochondrial oxygen consumption levels relative to the total respiratory output of the embryos.

The difference between the average oligomycin-resistant OCR and the azide-resistant, non-mitochondrial OCR reveals the level of proton leak across the inner mitochondrial membrane. Low levels of proton leak are indicative of high mitochondrial coupling and efficient ATP production (Brand and Nicholls, 2011; Jastroch et al., 2010). Compared to the low proton leak at 24 hpf for control animals (0.8 ± 0.5 pmol O_2 /min; $n=4$), DNP-treated embryos had greater than 20 times higher proton leak (19.3 ± 1.1 pmol O_2 /min, $n=4$; $p=0.03$; Fig. 1F). The average proton leak for the control embryos was 5.1 ± 2.0 pmol O_2 /min ($n=3$) at 48 hpf and 4.6 ± 6.5 pmol O_2 /min ($n=3$) at 72 hpf, which were not significantly different from the proton leak of DNP-treated embryos at either the 48 hpf (12.7 ± 4.8 pmol O_2 /min, $n=3$; $p=0.19$) or 72 hpf (11.5 ± 7.4 pmol O_2 /min; $n=3$; $p=0.38$). Although DNP treatment significantly alters proton leak and by extension efficiency of mitochondrial ATP production at 24 hpf, at later time points additional proton leak cannot be further induced in the DNP-treated embryos.

2.2. Chronic DNP exposure induces overall ATP and glycolytic deficiencies

Since DNP treatment impaired mitochondrial energy metabolism, we next investigated the energetic output of these embryos by measuring total ATP and lactate content. At 24 hpf, the total ATP content of DNP-treated embryos was 1.1 ± 0.2 pmol/ μ g protein ($n=7$) and not significantly different from the 0.8 ± 0.1 pmol/ μ g protein measured from control animals ($p=0.61$; $n=7$, Fig. 2A). By 48 hpf, the ATP level from DNP-treated embryos dropped to 0.9 ± 0.1 pmol/ μ g ($n=7$), significantly lower than the average ATP level of control embryos ($p=0.03$; 1.10 ± 0.1 pmol/ μ g, $n=7$; Fig. 2A). Between 24, 48, 72 and 96 hpf, ATP levels in control animals increased significantly each day ($p=0.03$, 0.03 , 0.003 , respectively) to 1.8 ± 0.2 pmol/ μ g ($n=7$) at 72 hpf and 3.5 ± 0.2 pmol/ μ g ($n=6$) at 96 hpf. In contrast, the levels of ATP in DNP-treated embryos at 96 hpf were not significantly different from their initial 24 hpf level ($p=0.4$). By 72 hpf, ATP levels in DNP treated embryos were just 1.0 ± 0.1 pmol/ μ g ($n=7$) and 1.5 ± 0.1 pmol/ μ g ($n=6$) at 96 hpf and at each time point were significantly lower than control values ($p=0.0073$ and $p=0.005$, respectively).

Levels of lactate, a major product of glycolysis, were measured to determine whether embryos with DNP-induced mitochondrial dysfunction used glycolysis to produce ATP (Fig. 2B). At 24 hpf, DNP-treated embryos had $\sim 4\times$ higher lactate levels than control embryos (26.3 ± 3.3 μ M, $n=6$ and 6.6 ± 1.7 μ M/embryo, $n=6$, respectively; $p=0.0051$). This high level of glycolysis was not maintained in the DNP-treated embryos at 48 hpf as the average control lactate level increased to 133.5 ± 19.8 μ M ($n=6$), significantly greater than 50.0 ± 14.8 μ M lactate/DNP-treated embryos ($n=6$; $p=0.0131$). Total lactate again increased at 72 hpf to 296.0 ± 48.7 μ M/control embryo ($n=6$), twice the 108.2 ± 13.7 μ M lactate/DNP-treated embryo ($n=5$; $p=0.0081$). data revealed that DNP-induced These mitochondrial dysfunction induced higher levels of glycolysis at 24 hpf compared to controls, but it was not sustained at later time points (Fig. 2B) where both ATP and lactate levels in the DNP-treated embryos were significantly lower.

2.3. Reactive oxygen species levels are attenuated by DNP exposure

We used two complementary methods to assess ROS, which are a normal byproduct of OXPHOS (Brand, 2000; Murphy, 2009). An immunoblot assay to detect protein carbonylation, a protein modification that may be induced by ROS oxidation, revealed that at 24 and 48 hpf, chronic DNP exposure significantly decreased the levels of protein oxidation when compared to control levels (Fig. 3A). At 24 hpf, the protein oxidation levels of whole DNP-treated embryos were $83.5 \pm 0.1\%$ of the controls ($p=0.0011$; $n=7$). DNP-treatment decreased protein oxidation to $73.5 \pm 0.1\%$ of control levels at 48 hpf ($p=0.0011$; $n=7$), before returning to control levels at 72 hpf ($p=0.36$; $n=4$). CM-H₂DCFDA, a fluorescein derivative that is non-fluorescent until cleaved by intracellular esterases and then oxidized, was used to assess oxidation levels in the heads of living zebrafish embryos (Anichtchik et al., 2008; Walker et al., 2012). H₂DCFDA levels of DNP-treated embryos were lower than in control embryos (Fig. 3B–F). At 24 hpf, the relative H₂DCFDA level of the DNP-treated embryos was 20% lower than controls ($81 \pm 3\%$, $p=0.007$; $n=25$ and 19, respectively). By 48 hpf, the relative H₂DCFDA level was $66 \pm 4\%$ compared to controls and significantly lower ($n=30$ and 14, respectively; $p=0.03$). The largest difference in relative H₂DCFDA fluorescence levels was detected at 72 hpf, where the levels of DNP-treated embryos were less than half that of controls ($43 \pm 1\%$; $n=12$ and $n=17$, respectively; $p=0.0009$). These assays of protein carbonylation and H₂DCFDA oxidation levels indicate that DNP-treated embryos had attenuated ROS levels.

Mitochondrial biogenesis is dependent upon the coordinated expression of mitochondrial and nuclear-encoded genes and is stimulated in response to changes in metabolic demands and stressors (for example, in response to exercise, injury, or oxidants) (Scarpulla, 2008). Because DNP significantly increased respiration in embryos beginning just 3 h after exposure (Fig. 1A), but continued DNP exposure significantly depressed respiration over the course of embryonic development (Fig. 1B), we hypothesized that DNP-treated embryos would induce mitochondrial biogenesis in an attempt to compensate for their OXPHOS inefficiencies. Quantification of mtDNA content with qPCR was conducted to estimate the mitochondrial number per cell. At 6 hpf, the mtDNA content of DNP-treated and control embryos was not different, but at 12 hpf DNP-exposure induced a 1.2 ± 0.2 fold increase in mtDNA content compared to that of control embryos ($n=3$, $p=0.02$), reaching its highest level at 24 hpf (1.8 ± 0.4 fold, $n=8$, $p=0.02$), remaining significantly higher than control levels at 48 hpf (1.3 ± 0.1 fold, $n=8$, $p<0.001$) and 72 hpf (1.1 ± 0.0 fold, $n=6$, $p=0.002$), before falling back to control levels at 96 hpf (Fig. 4A and Table 1).

mtDNA synthesis and gene expression of mitochondrial proteins are coordinately regulated in order to modulate OXPHOS capacity (Fernandez-Vizarra et al., 2011; Trounce, 2000). Because DNP exposure induced mtDNA synthesis (Fig. 4A), transcript levels of genes that contribute to mitochondrial biogenesis and quality control were quantified over the 96 h of embryonic development (Fig. 4B, C). Ten genes that fall into three broad categories were quantified: regulatory transcription factors/transcriptional activators (Fig. 4B and Table 1), genes involved in mitochondrial maintenance (Fig. 4C and Table 1), and genes that regulate and respond to oxidative stress (Fig. 4D and Table 1).

The expression of two of three transcription factors/transcriptional co-activators were significantly increased compared to control levels at 6 hpf: peroxisome proliferator-activated receptor γ coactivator 1a (*pgc1a*; 1.6 ± 0.2 fold, $n=6$, $p=0.005$), a key regulator of mitochondrial biogenesis (Ventura-Clapier et al., 2008), and the NF-E2 p45-related factor 2 (*nrf2*; 1.7 ± 0.1 fold, $n=6$, $p<0.001$), which regulates antioxidant and detoxification genes (Hayes and Dinkova-Kostova, 2014; Lee et al., 2005) (Fig. 4B). *pgc1a* and *nrf2* expression from DNP-treated embryos both dropped significantly below control levels at 12 hpf, with *pgc1a* levels staying below or equal to control transcript levels at between 12 and 96 hpf (Fig. 4B). At 48 hpf, *nrf2* transcript levels from DNP treated embryos jumped 2.1 ± 0.2 fold, significantly above control levels only to fall back to control levels at 72 and 96 hpf. Levels of hypoxia inducible factor-1 (*hif1a*), a transcription factor induced by changing oxygen levels and linked to the shift between OXPHOS and glycolysis (Brocato et al., 2014), were significantly different between DNP-treated and control fish only at 72 hpf when it showed a 1.2 ± 0.4 fold increase ($n=6$, $p=0.001$; Fig. 4B and Table 1).

All genes in the mitochondrial maintenance group had their highest levels at 6 hpf, and of those, 2 were significantly greater than controls; these included: optic atrophy 1, *opa1* (1.5 ± 0.1 fold, $n=6$, $p<0.001$), which encodes a protein involved in mitochondrial inner membrane fusion and cristae formation (Alexander et al., 2000; Delettre et al., 2000) and progressive external ophthalmoplegia, *peo1* (1.3 ± 0.1 fold; $n=6$, $p=0.007$), the sole mtDNA helicase whose expression has been linked to changes in mtDNA content (Spelbrink et al., 2001; Tyynismaa et al., 2004). The expression levels of these genes from the DNP-treated fish were significantly lower than controls at 12 and 24 hpf (Fig. 4C and Table 1). This gene group also included NADH dehydrogenase 5 (*nd5*), a mitochondrially-encoded component of OXPHOS Complex I machinery, and dynamin related protein 1 (*drp1*), a cytosolic GTPase that controls mitochondrial fission (Kageyama et al., 2011). At 48 hpf, *nd5* and *peo1* transcript levels from DNP-treated embryos were significantly greater than control fish (1.1 ± 0.1 fold, $p=0.003$ and 1.1 ± 0.1 fold, $p=0.01$ fold, respectively), but *opa1* and *drp1* expression did not differ from control levels. Again at 72 hpf, the expression of all four genes were significantly less than control levels from fish treated with DNP (Fig. 4C and Table 1). At 96 hpf, all genes in this group were equal to or significantly less than control levels (*nd5* decreased a $0. \pm 0 \pm 0.1$ fold, $p<0.001$).

We next quantified transcript levels of two genes involved in zebrafish ROS detoxification and DNA damage repair: 8-oxyguanine DNA glycosylase, (*ogg1*) which is regulated by *nrf2* (Hayes and Dinkova-Kostova, 2014) and recognizes and helps repair 8-oxoG DNA lesions (Singh et al., 2013), and *sod2*, the superoxide dismutase responsible for converting superoxide to H_2O_2 within the mitochondria (Flynn and Melov, 2013). At 6 hpf, *sod2* transcript levels from DNP-treated fish were significantly greater (1.3 ± 0.1 fold, $p=0.04$ and 1.1 ± 0.1 fold, $p=0.007$, respectively), and *ogg1* levels were significantly lower (0.9 ± 0.0 fold, $p<0.001$) than transcript levels from control animals (Fig. 4D and Table 1). DNP exposure significantly inhibited *sod2* transcript expression compared to control levels at all but the final time point, with the largest disparity occurring at 72 hpf where the expression level of DNP-treated embryos was just 0.6 ± 0.1 that of controls (Fig. 4D and Table 1). In contrast, the transcript levels of *ogg1* from DNP-treated animals showed the largest differences at 48

and 72 h (1.8 ± 0.2 fold, $p < 0.001$ and 1.5 ± 0.1 fold, $p < 0.001$) but were not different from control levels at 96 hpf. The significant decrease in *sod2* gene expression over time is consistent with the altered levels of embryonic ROS detected (Fig. 3). However, the *ogg1* expression was quite different than expected given the demonstrated decrease in embryonic ROS and corresponding decrease in *sod2* expression although this may be due to the transient increase in *nrf2* expression (see Section 3).

2.4. DNP exposure inhibits embryonic growth and decreases survival

Despite the DNP-induced decrease in ATP production and altered expression of genes associated with mitochondrial maintenance and functionality, many aspects of embryonic development and morpho-genesis proceeded normally (Fig. 5). This is particularly surprising because inhibiting the ATP synthase itself has been shown to arrest zebrafish embryos during gastrulation (Pinho et al., 2013). We found that early developmental events such as gastrulation, axis formation, somite formation, and early organogenesis proceeded normally in DNP-treated embryos (Fig. 5A–D versus Fig. 5E–H). At 24 hpf the effects of DNP were largely confined to reducing the embryonic growth rate, but in some embryos we observed that the boundaries of the brain regions appeared darker and were less distinct (Fig. 5E). The average body length of DNP-treated embryos was 2.08 ± 0.1 mm ($n=30$), 16% smaller than controls, which had reached 2.47 ± 0.0 mm ($n=30$; $p < 0.001$ Fig. 5I). The otic vesicle length ratio (OVL) is a measure of early development determined by dividing the distance between the otic vesicle and the eye by the length of the otic vesicle (Kimmel et al., 1995), which decreases as normal embryos age. In DNP-treated embryos the OVL was 6.27 ± 0.2 mm ($n=28$), significantly larger than the 5.23 ± 0.2 mm of control embryos ($n=34$, $p=0.0017$; Fig. 5J) at this time point. Although DNP-treated embryos have significantly shorter body lengths at 24 hpf, the average somite lengths were 58.3 ± 2.0 μ m ($n=25$) and 56.5 ± 2.4 μ m ($n=14$) for control and DNP-treated embryos, respectively ($p=0.52$; Fig. 5K). Survival of DNP-treated embryos was not significantly different from controls at 24 hpf ($75.5 \pm 4.0\%$ versus $74 \pm 4.3\%$, respectively, $p=0.7828$; Fig. 5L).

Continued DNP exposure, however, induced some abnormalities that are common to other zebrafish models of developmental disease (Pinho et al., 2013). For example, pericardial edema (arrow) formed in many of the embryos (arrow, Fig. 5F) and was maintained through 72 hpf (Fig. 5G and H). By 48 hpf, the average body length of embryos exposed to DNP was 13% percent smaller than control animals (2.54 ± 0.0 mm and 2.91 ± 0.0 mm, respectively), resulting in a significant difference ($n=27-28$; $p < 0.001$; Fig. 5I). DNP exposure also reduced the average somite length to 67 ± 3.3 μ m ($n=17$) compared to the 78.9 ± 2.9 μ m average from control embryos ($n=16$; $p=0.03$; Fig. 5K). At 48 hpf, the OVL (Fig. 5J) ratio of DNP-treated embryos (3.00 ± 0.2 , $n=34$) remained significantly larger than controls (0.69 ± 0.0 , $n=34$; $p < 0.001$). Despite these developmental abnormalities, the average survival of the control embryos ($69.8 \pm 5.3\%$, $n=14$) was not significantly different from the DNP-treated embryos ($72.3 \pm 4.4\%$, $n=14$; $p=0.8005$; Fig. 5L).

Between 48 hpf and 72 hpf, DNP treatment reduced survival by half. Only $34.8 \pm 6.1\%$ of DNP-treated embryos ($n=14$) reached the 72 hpf time point compared to $71.5 \pm 4.5\%$ ($n=14$) of the controls ($p=0.004$; Fig. 5L). The surviving DNP-treated embryos also had

significantly shorter total body length compared to controls (2.7 ± 0.1 mm and 3.1 ± 0.1 mm, $n=27$ respectively, $p < 0.001$; Fig. 5I). Likewise, at 74.6 ± 2.6 μ m ($n=15$), the average somite length of DNP treated embryos was significantly shorter than control fish (85.3 ± 2.1 μ m, $n=15$; $p=0.003$; Fig. 5K). Additionally, the OVL ratio remained significantly larger ($p < 0.001$) in DNP-treated embryos compared to controls (0.70 ± 0.0 , $n=34$ and 0.49 ± 0.0 , $n=37$, respectively; Fig. 5J). Together these data show that preceding the 48 hpf time point, DNP-exposed embryos have few phenotypic abnormalities, but by 48 hpf, continued DNP exposure produced significant developmental deficits and significantly affected survival.

2.5. Motor neuron development is disrupted in DNP-treated embryos

Compared to control animals, DNP-treated embryos were less responsive to experimental manipulations, which we assessed by quantifying the total movement of individual 72 hpf embryos within a 48 well plate. The fish were imaged at 1 min intervals for 27 min. Afterward, the differences between each consecutive image were calculated to reveal displacement. Fig. 6A shows the cumulative positions of individual fish per well, where a completely white well indicates a fish that remained stationary for the duration of the experiment. These results reveal that while all control fish (16/16) moved at least once (rows 1–2, Fig. 6A), only 10/32 DNP-treated fish (rows 3–6, Fig. 6A) moved from their starting positions in the well. We calculated the percent of each well masked by the cumulative positions of each fish as an indicator of movement and found that at $9.9 \pm 1.5\%$, the control animals moved significantly more than the DNP-treated fish ($3.2 \pm 0.3\%$; $p < 0.0001$).

Given that the DNP-treated embryos exhibited less spontaneous movement than control animals, we next investigated whether DNP affected somite development by using an antibody to detect the engrailed-expressing muscle pioneer cells, a population of precursor cells that are necessary for the proper organization of the myotome (Yin and Solnica-Krezel, 2007). Using the 4d9 antibody to label the muscle pioneer cells, we did not detect differences in the position or number of the cells along the myoseptum (Fig. 7A–D). At 24 hpf, control animals had an average of 3.43 ± 0.3 ($n=10$) labeled muscle pioneer cells, compared to 3.4 ± 0.3 ($n=6$) measured from DNP-treated embryos (Fig. 7D), suggesting that early development of the myotome was not disrupted by DNP. Likewise, the dorsal–ventral diameter of the neural tube did not differ at any time point between the control and DNP-treated embryos. At 24 hpf, the neural tube diameter of control embryos was 55.2 ± 2.4 μ m ($n=16$) versus 53.7 ± 3.3 μ m for DNP-treated embryos ($n=9$; $p=0.43$). At 48 hpf, the diameter of control animals was 55.3 ± 3.2 μ m ($n=8$) versus the 52.0 ± 2.0 μ m ($n=7$) diameter of DNP-treated embryos ($p=0.41$), and at 72 hpf the control diameter reached 59.6 ± 1.3 μ m ($n=12$), which was not significantly different from the DNP-treated embryo neural tube diameter (60.4 ± 2.4 μ m, $n=14$; $p=0.43$). Lastly, we used confocal microscopy to examine the 3a10 neurofilament antibody-labeled Mauthner neurons of 17 control and 13 DNP-treated embryos between 48 and 57 hpf. The Mauthner neurons are the cells responsible for governing the escape response swim behaviors of zebrafish (Korn and Faber, 2005). Our analysis revealed that these cells were present and the axon arbors were similar in both DNP-treated and control animals (Figs. 7F–H). Because these different markers of the development of the motor system were not perturbed by DNP exposure, these data

suggested that the development of the motor neurons themselves may be affected by DNP exposure.

Each somite of the tail contains a small number of primary motor neurons that exit the neural tube; by 24 hpf they have yet to elaborate their full axonal arbor, but have begun to make synaptic contacts with the tail musculature (Flanagan-Steet and Steet, 2013; Meyers et al., 1986). We used the *znpl/zn1* monoclonal antibody combination to label the motor neurons (Fig. 8A–D) and measured the length of the primary axon of the ventral projecting Caudal Primary (CaP) motor neurons over the first 3 days of development. In control animals at 48 hpf, the CaP axon has projected ventrally toward the yolk extension before turning dorsolaterally (arrows, Fig. 8A and diagram in B). In contrast, the CaP axons of the DNP-treated embryos grow ventrally but are truncated (arrows, Fig. 8C, D). Another striking difference between the control and DNP-treated embryos was the minimal axonal branching that is easily visible in the 90° rotated projection of the somite of the control animal, but largely absent in the animal with DNP-induced mitochondrial dysfunction (Fig. 8B versus D). The primary CaP axon was reconstructed from where it exited the neural tube to its most distal point and quantified the average axon length of control and DNP-treated embryos for 24, 48 and 72 hpf. At 24 hpf, the average control axon length was $89.1 \pm 7.6 \mu\text{m}$ ($n=11$), and was significantly longer than the $60.1 \pm 8.7 \mu\text{m}$ ($n=10$) axon of DNP-treated embryos ($p=0.04$; length Fig. 8E). The CaP axon development continued to be inhibited by DNP, reaching only $130.4 \pm 18.9 \mu\text{m}$ ($n=7$) at 48 hpf and $174.3 \pm 18.6 \mu\text{m}$ ($n=7$) at 72 hpf. In contrast, the average CaP axon length of the control embryos was $271.0 \pm 15.3 \mu\text{m}$ ($n=5$) at 48 hpf and $350.9 \pm 11.1 \mu\text{m}$ ($n=7$) at 72 hpf, each significantly greater than the CaP length of DNP-treated embryos ($p=0.005$ and $p=0.002$, respectively; Fig. 8E). Taken together, these data indicate that DNP exposure did not produce significant changes in the early development of motor system structures, such as formation of the neural tube, somites and Mauthner cell circuitry. We found that DNP instead inhibited motor neuron axon development during late embryogenesis.

2.6. DNP exposure disrupts eye development

Optic neuropathy is often associated with mitochondrial disease (Maresca et al., 2013) and examination of treated embryos revealed that the development of the eye was altered by chronic DNP exposure. At 24 hpf, the rostral–caudal diameter of the eyes of control and DNP-treated embryos were not significantly different ($p=0.19$) with the eyes of control embryos reaching $193.4 \pm 5.0 \mu\text{m}$ ($n=39$) and $180.1 \pm 7.8 \mu\text{m}$ ($n=23$) for DNP-treated embryos (Fig. 9A, C, F). At this stage, the average lens diameter of DNP-treated embryos was $67.2 \pm 1.9 \mu\text{m}$, which was also not different from controls ($67.3 \pm 2.6 \mu\text{m}$, $p=0.97$; black portion of bars, Fig. 9A).

By 48 hpf, the average eye diameter of control embryos had grown to $315.6 \pm 10.7 \mu\text{m}$ ($n=36$) and their lenses reached $105.3 \pm 4.5 \mu\text{m}$ (Fig. 9A, D). In contrast, the eyes of 48 hpf embryos exposed to DNP reached a diameter of $208.4 \pm 8.2 \mu\text{m}$ ($p<0.0001$; $n=38$) and their lens diameter was $73.4 \pm 2.8 \mu\text{m}$ ($p=0.0001$; Fig. 9A, G). At 72 hpf, the total diameter of the eyes of control animals had reached $315.6 \pm 10.7 \mu\text{m}$ and the average lens diameter was $102.1 \pm 4.4 \mu\text{m}$ ($n=19$). With an average eye diameter of $220.3 \pm 8.1 \mu\text{m}$ and lens of $79.0 \pm 3.0 \mu\text{m}$ ($n=37$),

the DNP-treated embryos continued to have significantly smaller total eye and lens diameters compared to controls (both $p < 0.0001$; Fig. 9A, E, H).

We next investigated whether the decreased eye size could in part be caused by increased cell death. In vivo two-photon stacks were acquired through the eyes of embryos labeled with acridine orange (AO), a fluorescent marker that penetrates and labels the nuclei of dying cells (Furutani-Seiki et al., 1996; Li et al., 2000). At 24 hpf, DNP exposure did not significantly alter the normal level of cell death that is associated with the development of the eye ($p = 0.17$; (Cole and Ross, 2001). Compared to control embryos, which had 69.1 ± 9.9 AO-positive nuclei per eye ($n = 15$), the eyes of DNP-treated embryos had 50.57 ± 3 AO-labeled nuclei ($n = 13$; Fig. 9B, C, F). When levels of cell death within just the lens were examined at 24 hpf, however, DNP-treated embryos had significantly fewer AO-positive nuclei (13.7 ± 9.2 ; $p = 0.02$) compared to 26.3 ± 16.2 AO-labeled nuclei of control lenses (black portion of bars, Fig. 9B). By 48 hpf, the total number of AO-labeled nuclei within the eyes of the DNP-treated embryos was 51.5 ± 8.0 ($n = 14$; $p = 0.01$), significantly greater than the 25.0 ± 3.3 labeled nuclei in control eyes ($n = 18$; Fig. 9B, D, G). Within just the lens, the average number of AO-labeled nuclei of the control embryos dropped to just 4.7 ± 5.6 , significantly fewer than the 18.4 ± 17.8 found in the lens of DNP-treated embryos ($p = 0.007$). This increased level of DNP-induced cell death in the eye continued, and at 72 hpf there were 94.7 ± 18.8 AO-positive nuclei in the DNP-treated animals, significantly greater ($p = 0.01$; $n = 10$) than the 32.4 ± 4.6 AO-labeled nuclei of control embryos ($n = 7$; Fig. 9B, E, H). At this stage, control animals had significantly fewer AO-positive nuclei in their lenses (0.9 ± 1.2) compared to the 12.7 ± 12.6 AO-labeled cells found in the lenses of DNP-treated embryos ($p = 0.01$). These data indicate that initially DNP may provide a slight cytoprotective action in the developing eye that is lost by 48 hpf. Alternatively, the decrease in AO-positive nuclei upon DNP treatment may indicate a general effect of DNP on eye development as programmed cell death is an important part of normal eye formation (Cole and Ross, 2001).

Because DNP exposure interfered with the growth of the eye, we then focused on the development of the retina itself. The neural circuitry of the retina is organized into alternating layers of neuronal cell bodies and layers of synaptic connections that are free from neuronal cell bodies (Mumm et al., 2005). The nuclear stain Sytox Red was used to reveal the cell body-containing layers of the retina: the ganglion cell layer (GCL), the inner nuclear layer (INL) and the photoreceptor layer (PL). The antibody against synaptic vesicle protein 2 (SV2) was used to label the synapse-rich inner plexiform layer and outer plexiform layers (IPL and OPL; Fig. 10A–D). In control embryos, the retina develops quickly (Schmitt and Dowling, 1994) and by 72 hpf retinal laminae have formed (Fig. 10A, B). The OPL, which is made up of the connections between photoreceptors and the INL neurons, is clearly labeled by the SV2 antibody as is the thick IPL that is formed by the concentration of synaptic contacts between the retinal ganglion cells (RGCs) and the neurons within the IPL (Fig. 10A, B). Axons of all the RGCs exit the eye together and form the optic nerve (Fig. 10B), which is also labeled by the SV2 antibody. In contrast to the eyes of control animals, the retinal laminae are largely absent in the eyes of DNP-treated embryos, with only faint evidence that the nuclei of the GCL and the INL have separated by diffuse and disorganized SV2 labeling of their synaptic connections of the INL (Fig. 10C and asterisk in D). The

optic nerve of the DNP-treated embryos, though present, was less developed when compared to control embryos (Fig. 10D).

The smaller retinal IPL and altered optic nerve of DNP-treated embryos suggests that the development of the ganglion cells, among the first neurons to differentiate in the retina, may be affected by exposure to DNP. The POU domain transcription factor *Brn3a* (*pou4f1*) is expressed in select primary sensory neurons and neurons of the central nervous system, including the RGCs. The GFP transgenic zebrafish line *Tg(brn3a-hsp70:GFP)* was used to visualize the RGCs in control and DNP-treated embryos (Fig. 10E–H). While the RGCs of the control embryos were distributed into a coherent and dense GCL (Fig. 10F), DNP exposure decreased the number of RGCs generated and disrupted the formation of the GCL (Fig. 10G–L). We found examples of DNP-treated embryos with no detectable *Brn3a:GFP*-expressing cells in the retina (Fig. 10G, H). More commonly, we found fewer GFP-labeled RGCs (Fig. 10I–J) and GCL disorganization, with labeled cells appearing outside the GCL (asterisk, Fig. 10L). Together these data show that DNP decreased the number of RGCs generated, decreased the ability of the eye to produce coherent retinal laminae and altered the balance of cell death expected in normal eye development.

3. Discussion

In order to gain a better understanding of progression of mitochondrial disease during development, we have used a low concentration of DNP to induce systemic mitochondrial dysfunction in the developing zebrafish embryo. By combining methods to assess bioenergetics, the abundance of mtDNA, the levels of transcripts involved in mitochondrial function, and the altered development of the embryo and its nervous system, we provide for the first time a cellular and molecular profile of mitochondrial function and dysfunction over the course of embryonic development. The nervous system is a common source of pathology in pediatric mitochondrial disease (Koenig, 2008), and by focusing our analysis on the development of two well-described aspects of nervous system development in zebrafish, that of the retina and primary motor neurons, we quantify at the level of single, identifiable neurons and neural circuits how mitochondrial dysfunction alters neurodevelopment. Together these data provide insights into the complex relationship between mitochondrial homeostasis and embryonic development, as well as the pathophysiology and progression of congenital mitochondrial disease. This study builds on our previous work where we developed a method for measuring oxygen consumption in a whole living zebrafish embryo over the course of early development (Stackley et al., 2011) and examined the role of the mitochondrial fission protein Opa1 in zebrafish development (Rahn et al., 2013).

The first goal of this study was to describe the time course of DNP's actions on mitochondrial output. Previous studies have described the use of DNP on zebrafish in order to study swimming performance and triglyceride levels (Marit and Weber, 2011) and as a control toxicant in a study of transcriptional responses to compounds (Kluver et al., 2011). Marit and Weber (2011) utilized adult zebrafish treated with 6 and 12 mg/L DNP for only 24 h and demonstrated reduced swim speed and changes in triglyceride levels while Kluver et al. (2011) treated embryonic fish at either 2 or 24 hpf for either 24 or 48 hpf with 14 μ M DNP and examined gene expression profiles compared to their main test compounds. Both

of these studies describe treating zebrafish at much higher doses than we found to be effective (here we describe treatment at 0.5 μM or 0.09 mg/L). Rather than focusing on the toxic effects of DNP, our study used very low doses for longer periods of time in order to model mitochondrial dysfunction in particular. Importantly, using our established methodologies we were able to examine the complex effects of this dysfunction on whole embryo respiration.

After only 3 h of DNP exposure, in vivo respirometry showed that DNP treatment significantly increased basal cellular respiration (Fig. 1A). DNP acts to disrupt the proton gradient resulting in decreased mitochondrial ATP output. Cells attempt to compensate for decreased ATP production by increasing cellular respiration (Lou et al., 2007), which underlies the increased oxygen consumption we observed at 6 hpf (Fig. 1A). Further pharmacological dissection of mitochondrial output over the course of embryonic development revealed that while the basal respiration rates had fallen back to control levels at 24 hpf (Fig. 1B), the DNP-treated embryos had very high levels of proton leak (Fig. 1F) consistent with the known action of DNP as a proton ionophore (Lou et al., 2007). Proton leak across the mitochondrial matrix results in inefficient ATP production (Jastroch et al., 2010), detected as significantly lower mitochondrial ATP-linked respiration rates in DNP-treated embryos (Fig. 1D).

DNP also decreased non-mitochondrial respiration compared to control levels throughout most of embryonic development (Fig. 1E). However, if we consider the overall oxygen consumption of the DNP-treated embryos, we observed that the proportion of total respiration that is due to non-mitochondrial respiration was not different between controls and DNP-exposure. Nonetheless, it is important to consider the potential actions of DNP on non-mitochondrial respiration. Oxygen consumed by non-mitochondrial oxidases makes up non-mitochondrial respiration (Bishop and Brand, 2000; Harper and Brand, 1993). It is not clear which cellular oxidases in particular contribute to non-mitochondrial respiration, but oxidases within endosomes, lysosomes, peroxisomes and the endoplasmic reticulum, for example, may make up the bulk of non-mitochondrial oxygen consumption (Bishop and Brand, 2000; Harper and Brand, 1993). To our knowledge, no oxidase has been described to be a direct target of DNP. However, the effects of DNP and other proton ionophores on cell or organelle membrane potential and pH have been reported; an action which may alter oxidase activity. For example, 100 μM DNP was the minimum dose capable of depolarizing isolated carotid body cells, and 250 μM was necessary to decrease their pH by 0.1 unit (Buckler and Vaughnan-Jones, 1998). 2 mM DNP disrupted vacuolar H^+ -ATPase proton pump activity and altered the pH of yeast (Dechant et al., 2010). Hudman et al. (2002) found that 200 μM DNP induced a significant increase in the intracellular calcium of cardiac myocytes, in part due to the drug liberating calcium from the sarcoplasmic reticulum. In a recent study that examined the effects of DNP on a variety of targets, 10 μM DNP was necessary to alter the pH and 1 μM DNP was necessary to induce significant changes in the voltage measured across an artificial bilayer lipid membrane, while 10 μM DNP was the minimum dose that significantly altered the membrane potential of yeast (Antonenko et al., 2013).

We have not ruled out the possibility that DNP may be acting directly on these non-mitochondrial targets in the zebrafish embryos. It is likely, however, that these DNP actions are minimal given that the dose applied to the intact animals (0.5 μ M) was many folds lower than what was used in the previous in vitro studies, and DNP exposure did not significantly decrease the proportion of OCR contributed by non-mitochondrial respiration. Instead, the DNP-induced decrease in non-mitochondrial respiration may result from the mitochondrial dysfunction it causes. The proton pumps that maintain organellar pH and ion gradients are among the most ATP-demanding complexes of developing animals and their nervous systems (Lennie, 2003), and these pH and ion gradients are important for oxidase activity (Diering and Numata, 2014; Mellman, 1992; Orłowski and Grinstein, 2007). In light of this, the observed decrease in ATP production driven by DNP treatment may be inhibiting the activity of the ATP-dependent proton pumps, thereby altering the pH or ion gradients and ultimately driving the lower non-mitochondrial respiration we document. In addition, levels of non-mitochondrial oxidase activity have also been linked to mitochondrial ROS production, (Maynard et al., 2013), an observation in line with our data indicating decreased ROS production as a result of DNP treatment (see below). Despite the decrease in mitochondrial ATP output, at 24 hpf the total organismal ATP content of DNP-treated embryos was not different from control embryos (Fig. 2A). The significantly higher levels of lactate, a product of glycolysis, indicated that the embryos with mitochondrial dysfunction are compensating for their decreased OXPHOS efficiency and ATP output by significantly increasing glycolysis (Fig. 2B), resulting in the normal levels of total ATP content at 24 hpf (Fig. 2A). The ability of the embryos to make quick use of glycolysis in order to compensate for reduced OXPHOS capacity is likely aided by the metabolic plasticity of tissues in early stages of differentiation (Facucho-Oliveira and St John, 2009). For example, glycolysis is favored by the proliferating cells of the eye of *Xenopus laevis* which shift to OXPHOS after differentiation (Agathocleous and Harris, 2013). However, the initial ability of the embryos to compensate for the OXPHOS deficiencies by increasing glycolysis did not extend past 24 hpf. By 72 hpf, ATP-linked respiration rate (Fig. 1D) and levels of available ATP (Fig. 2A) were decreased compared to control embryos.

When we next examined the expression of several key genes, we were struck by how the transcript levels of many of the genes we examined shared similar profiles in response to DNP treatment. All but two genes from our panel (*hif1a* and *ogg1*) displayed relatively high levels three hours after DNP exposure (6 hpf). Many of genes we monitored contribute to mitochondrial biogenesis (Hock and Kralli, 2009), but the increased expression of *pgc1a* is especially significant as it is considered the key transcriptional co-activator of mitochondrial biogenesis genes (Lin et al., 2005). Indeed, mitochondrial biogenesis was induced as mtDNA content began to increase at 12 hpf reaching its peak at 24 hpf likely in response to this initial increase in *pgc1a* expression. The transcript levels of all genes on the panel fell to equal to or less than control levels at 12 hpf, with most remaining near control levels throughout embryogenesis (Fig. 4C, Table 1). After reaching its highest levels at 24 hpf (1.8-fold increase compared to control mtDNA levels), the mtDNA levels of DNP-treated embryos also fell over the course of development reaching control levels after 72 hpf (Fig. 4A). These data indicate that early on, DNP-treated embryos induced mitochondrial

biogenesis but their attempt to counter the mitochondrial dysfunction caused by DNP were not sustained at later stages.

Our next objective was to examine ROS levels and indicators of mitochondrial stress in the embryos. As the majority of cellular ROS are formed as a byproduct of OXPHOS, mitochondrial dysfunction is often accompanied by altered ROS levels (Liu et al., 2002). Work in mice has demonstrated that DNP treatment results in lowered ROS production (Caldeira da Silva et al., 2008). Mechanistically, uncoupling can decrease ROS release by creating lower oxygen tensions in the mitochondria decreasing the likelihood of superoxide formation (Balaban et al., 2005). Uncoupling could also decrease ROS production by lowering membrane potential and therefore impact the probability of reverse electron transfer from complexes II to I (Turrens, 2003). Based on these observations, and given respiration rates were lower in the DNP-treated embryos (Fig. 1), we hypothesized that treatment of zebrafish embryos with DNP would result in decreased ROS production.

We used two methods to estimate ROS levels in the embryos: protein immunoblots from whole embryos and the H₂DCFDA fluorescent reporter within embryo heads (Fig. 3). Both methods revealed that the levels of ROS was decreased at 24 and 48 hpf, but only the H₂DCFDA reporter indicated decreased ROS DNP-treated embryos at the 72 hpf time point. This difference may be due to the type of tissue analyzed in each assay. The immunoblot method was performed on whole embryo lysates while the H₂DCFDA assay focused only on embryo heads, which are composed mainly of nervous system tissue. It is possible that as the embryo approaches the larval stage around 72 hpf and tissues become more functional, tissue-specific differences in the production and removal of ROS develop. The observed difference between the two assays may also be due to the fundamental nature of the assays themselves. While both assays are commonly used to measure ROS levels, they do so in different ways. The immunoblot method measures protein carbonylation, which can result from action of ROS on specific amino acid residues. However, there is evidence that carbonylation can result from processes other than those related to ROS and may be affected by artificial factors like nucleic acid contamination (Levine et al., 2000; Luo and Wehr, 2009; Wang and Powell, 2010). Furthermore, the H₂DCFDA fluorescence measure, which is often attributed solely to hydrogen peroxide, in fact requires the presence of mitochondrial cytochrome c or Fenton-type reactions (Karlsson et al., 2010).

In addition to our two basic measures of ROS, we also aimed to examine expression of key genes as a way to describe the effect of DNP on downstream ROS targets. The qPCR profile of the mitochondrial dismutase, *sod2*, revealed that after a slight increase at 6 hpf, expression fell below control levels throughout most of embryonic development (Fig. 4D and Table 1). These data indicated that the DNP-induced decrease in ROS resulted in down-regulation of *sod2* as less detoxification was required by the embryos. Transcript levels of *ogg1*, a gene involved in repair of nuclear and mtDNA damage, were also significantly decreased during the first 24 h of development of DNP-treated embryos (Fig. 4D) before increasing significantly at 48 and 72 hpf. We originally hypothesized that DNP treatment and consequent reduction in ROS would result in a down-regulation of *ogg1* expression presumably because fewer oxidative lesions requiring the attention of DNA repair processes would be created. This observed 1.5-fold increase in *ogg1* expression at 48 and 72 hpf was

surprising and is perhaps due to the concomitant increase in *nrf2* which showed a marked 2-fold increase at 48 hpf (Fig. 4B). Nrf2 is a transcription factor that regulates the expression of over 200 genes that are largely involved in combating oxidative stress, and recent data suggests that *ogg1* is an Nrf2 target (Hayes and Dinkova-Kostova, 2014; Singh et al., 2013). We also found the fluctuating levels of *nrf2* surprising. DNP exhibited a positive response in a chemical screen used to find compounds that trigger the Nrf2/antioxidant response element (ARE) signaling pathway (PubChem, 2014). With that in mind, it is possible that this fluctuation in *nrf2* expression is due to the embryo responding to DNP as a toxicant. However, the amount of DNP we used in these studies was quite low and one might expect the levels of *nrf2* to be steadily increased over time if simply resulting from exposure to the chemical.

These data may suggest that DNP exposure results in conflicting cellular messages. On the one hand, DNP attenuates embryonic ROS levels and levels of *sod2*, implying a less stressed cellular state, yet at the same time induces *nrf2* and *ogg1* expression, known indicators of cellular stress. However, it is also important to consider that Nrf2 and its family members are also essential regulators of embryonic development, controlling erythropoiesis and angiogenesis (Shivdasani and Orkin, 1995; Wei et al., 2013; Williams et al., 2013). Likewise, Ogg1 has recently been shown to regulate embryonic cell proliferation and differentiation (Reis and Hermanson, 2012; Yan et al., 2013). In addition, one possible explanation may lie with ROS itself. While lower oxidative stress may be beneficial, ROS are also well described signaling molecules that regulate normal embryonic (Dennery, 2007; Finkel, 2011) and nervous system development (Celotto et al., 2012; Tsatmali et al., 2005; Valero et al., 2012). Therefore, changes in gene expression or in the levels of ROS by DNP may alter signaling cascades that are normally regulated by these molecules during development and may contribute to the phenotypic abnormalities or gene expression changes that we describe. Alternatively, other non-ROS signaling pathways controlled by mitochondria such as altered cytoplasmic calcium levels (Hudman et al., 2002) may be causing the cellular stress indicated by the increased expression of *nrf2* and *ogg1*. Further experiments are needed to elucidate the mechanisms and biological significance of these changes.

After assessing several physiological and molecular indicators of mitochondrial dysfunction, we turned to examine the development of DNP-exposed zebrafish embryos, capitalizing on the ability of our system to link such dysfunction to developmental abnormalities in real time. Despite the high level of mitochondrial dysfunction that we detected physiologically (Fig. 1) and molecularly (Fig. 4) within hours of DNP exposure, it was surprising that the main phenotype we observed during early embryogenesis was evidence of slower development compared to controls (Fig. 5). Most complex early developmental events such as body axis formation, segmentation, and organ differentiation proceeded normally in DNP-treated embryos. However, the continued exposure to DNP severely affected later developmental events. By 48 hpf, DNP-treated embryos were significantly smaller than the untreated control animals, and had begun to show evidence of cardiac edema (Fig. 5F). At 72 hpf, DNP-induced mitochondrial dysfunction resulted in significantly lower whole embryo size and increased mortality compared to controls.

Because mitochondrial disease in children is often associated with neuropathies (Haas et al., 2007; Koenig, 2008), we focused our quantitative analysis of the effects of embryonic mitochondrial dysfunction on two well-characterized cell populations in neurodevelopment of zebrafish embryos: the axon outgrowth of the CaP primary motor neurons (Beattie et al., 2007) and the differentiation and development of the retina (Mumm et al., 2006). DNP exposure did not significantly alter the development of the lens and retina until later in embryonic development (Figs. 9 and 10). In fact, we found that DNP treatment reduced the normal level of apoptosis at 24 hpf. Sustained mitochondrial dysfunction results in older embryos with fewer RGCs (Fig. 10I–L) and less defined optic nerves (Fig. 10D) compared to controls (Fig. 10B). These eye defects are not unlike the optic atrophy common to mitochondrial disorders (Carelli et al., 2009; Yu-Wai-Man et al., 2011). The effects of embryonic DNP exposure on eye development are not limited to just the RGCs. The smaller eye size (Fig. 9A) and absence of retinal laminae (Fig. 10) suggests that the DNP-treated embryos lack a differentiated and fully functional photoreceptor layer and the processing neurons of the inner nuclear layer circuitry. Because these layers can develop in the absence of RGCs (Kay et al., 2004), DNP must also be acting on the other cell types of the retina.

In human subjects, DNP exposure has been associated with dysfunction of the peripheral nervous system (Nadler, 1935; Phillips and Singer, 2013), and peripheral neuropathy is also a common feature of mitochondrial disease in children and adults (Katsetos et al., 2013; Menezes and Ouvrier, 2012). Yet, DNP has shown therapeutic potential in models of stroke (Mattiasson et al., 2003), focal ischemia (Korde et al., 2005), recovery after sciatic nerve crush (da Costa et al., 2010), and traumatic brain injury (Pandya et al., 2007). In zebrafish embryos, DNP-induced mitochondrial dysfunction inhibited CaP motor neuron axon outgrowth and decreased the complexity of the CaP and other ventrally-projecting axonal arbors (Fig. 8). Although CaP axon length was inhibited by DNP at 24 hpf, we found the most profound differences at the later time points and by 72 hpf the CaP axons of the DNP-treated embryos were less than half the length of control embryos (Fig. 8). The developing neuromuscular system of zebrafish has revealed mechanistic insights into many neuromuscular diseases (McGown et al., 2013; Muller et al., 2010; Steele et al., 2014) and is likely to provide important information linking our observations with neuromuscular pathology observed in patients with mitochondrial diseases.

Improving our understanding of how mitochondrial dysfunction reveals itself during development is necessary for advancing potential diagnostics and therapies. Currently there are no treatments for mitochondrial disease and diagnostic methods often lack the sensitivity to detect pediatric mitochondrial disease in its early stages (Koenig, 2008). For example, the common diagnostic tool, venous lactate levels, provides limited diagnostic accuracy in children (Munnich et al., 1996). Our data demonstrates that the lactate levels only increase transiently in response to mitochondrial dysfunction, perhaps indicating that this diagnostic tool may only be relevant at a particular stage of disease development. Additionally, our data emphasize the utility of this whole animal model for examining the complex interactions of mitochondrial energetics and development. In addition to helping focus the development of diagnostics, given that DNP-induced dysfunction produced quantifiable changes in embryonic mortality and measures of neurodevelopment, this whole organism

model will be used for the discovery of therapeutics targeting mitochondrial dysfunction (Zon and Peterson, 2005).

4. Experimental procedures

4.1. Animals

Wild-type zebrafish (*Danio rerio*, AB strain) were obtained from the Zebrafish International Resource Center, which is supported by grant P40 RR012546 from the NIH-NCRR. *Tg(brn3a-hsp70:GFP)* fish were acquired from RIKEN, Japan (Aizawa et al., 2005). All zebrafish were maintained at 28.5 °C with a 14/10 h light/dark cycle and crossed according to standard methods (Westerfield, 2007). Embryos were staged using the criteria of Kimmel et al. 1995. Animals used for imaging experiments were treated with 0.75 mM phenylthiourea beginning at 22 h post-fertilization (hpf) to inhibit pigmentation. Animal studies were approved by the Medical University of South Carolina Institutional Animal Care and Use Committee (AR 2850) and performed in accordance with the defined guidelines.

4.2. DNP exposure and survival analysis

At 3 hpf, approximately 50 embryos were transferred to each well of a 6 well tissue culture plate filled with 5 mL of E3 medium (5 mM NaCl, 0.15 mM KCl, 0.33 mM CaCl₂, 0.33 mM MgSO₄. 2,4-dinitrophenol (DNP, Sigma, St. Louis, MO) dissolved in DMSO was added to the well to yield a final concentration of 0.5 µM DNP and 0.05% DMSO. Control animals were treated with 0.05% DMSO only. The daily survival rates were calculated by counting the number of embryos (~4569 embryos from 14 embryo clutches) still alive (as defined by having an active heartbeat) at 24, 48 and 72 hpf.

4.3. Quantification of development

The development and survival rates of the embryos was observed using a Zeiss Axio Observer.A1 microscope equipped with a 2.5× and 10× objective and AxioCam HRc CCD camera to acquire brightfield images. Standard length and otic vesicle length (OVL) was determined as described in Kimmel et al. (1995).

4.4. ATP assay

ATP concentrations were determined using a fluorometric ATP assay (BioVision, Milpitas, California). Embryos (10 per time point) were combined with 50 µL ATP assay buffer, homogenized using microfuge pestles and a motorized homogenizer and centrifuged at 15,000g for 2 min at 4 °C. Protein concentrations of the super-natant were determined using the bicinchoninic acid assay (BCA; Sigma, St. Louis, MO). 20 µL of total protein lysate was used for ATP determination. Cellular concentrations of ATP were determined using a standard curve of known ATP concentrations and adjusted for total protein in µg of cellular lysate.

4.5. Lactate assay

Pools of 10 embryos were collected at each time point and were homogenized in 75 mL cold Triton X-100 buffer (150 mM NaCl, 50 mM Tris HCl pH 7.2, 1% Triton X-100). Tubes were incubated for 10 min at 4 °C, and then centrifuged at 12,000g at 4 °C for 10 min. Lactate analysis was performed from the supernatant on all samples according to Brandt et al. (1980). All reagents were procured from Sigma-Aldrich and solutions made fresh prior to the assay.

4.6. Protein extraction and Oxyblot analysis

Protein lysates from 20 devolged embryos were prepared in 35 µL RIPA buffer (150 mM NaCl, 1 mM EDTA, 50 mM Tris HCl pH 7.5, 1% NP-40) supplemented with 1:100 protease inhibitor cocktail (Sigma P8340, St. Louis, MO) and homogenized as above. Samples were centrifuged at 4 °C for 10 min and the supernatant removed to a new tube. Concentrations were determined using the BCA assay on a 1:10 dilution of each sample. Oxidized protein levels were determined using the Oxyblot kit (Millipore, Billerica, MA).

4.7. DNA extraction and mtDNA copy number analysis

Pools of 20 embryos were incubated overnight at 55 °C in 250 µL digestion solution (50 mM Tris-HCl pH 8.0, 200 mM NaCl, 100 mM EDTA pH 8.0, 1% SDS, 0.2% DTT, 2 mg/mL proteinase K (EMD Chemicals, Billerica, MA). DNA was prepared using phenol chloroform extraction (Sambrook and Russell, 2001) and quantified by PicoGreen assay (Invitrogen, Grand Island, NY). 8.5 ng of DNA was used in a qPCR reaction using SsoAdvanced SYBR Green Supermix (BioRad, Hercules, CA) with appropriate primers (300 mM each) using the BioRad CFX96 RealTime instrument. Primers were designed to amplify nuclear DNA and mtDNA. Elongation 1-alpha (*efla*) was selected as the nuclear gene and mtDNA was measured with primers specific for the mitochondrially encoded NADH dehydrogenase-1 (*nd1*). The following cycle conditions were used: 95 °C/3 min, 30 cycles of 95 °C/15 s, 62 °C/30 s, followed by 95 °C 30 s and a dissociation curve. Samples were run in duplicate and averaged for each run individually. Primer pairs were verified and 95–105% efficiency confirmed by the standard curve method. The mtDNA fold changes, t-tests and p-values were calculated using the Bio-Rad CFX Manager 3.1 software, which uses the normalized expression calculation (ddCt) of Livak, Schmittgen (2001). Samples were normalized to *efla* Cts and are shown as fold change differences from control embryos.

Primer sequences (5'–3'):

efla F: AAGCCGCTGAGGTAAGCGTTCAAC

efla R: TTGAGCCGAGAAACGCGTGCTG

nd1 F: GGGCACCCATACCCATGCCCTAT

nd1 R: TGCGCTACAGCTCGTAAGGC

4.8. RNA extraction and gene expression analysis

RNA was extracted from 20 embryos for all time points using Trizol (Invitrogen, Grand Island, NY) followed by RNeasy Mini Kit (Qiagen). Embryos were pooled at the time of collection and all water removed before being flash frozen at -80°C. Frozen embryos were homogenized in 800 µL Trizol using in-tube pestles and a motorized homogenizer. Following a 5-min incubation at room temperature, 200 µL chloroform was added and the samples separated by centrifugation at 12,000g for 10 min. The aqueous phase was transferred to a new tube, 250 µL 100% ethanol was added and the samples mixed before proceeding with Qiagen mini-column RNA extraction. Samples were eluted in 30 µL RNase free water and quantified using a NanoDrop (Thermo) instrument. Only RNA of sufficient quality (OD 260/280 2.0–2.4 and OD 260/230 1.8–2.2) was used to prepare cDNA using the RETRO-script kit (Ambion) with 500 ng total RNA. cDNA was then diluted 1:1 with sterile dH₂O and 2.5 µL were used in a qPCR reaction as described above. The same PCR cycling program was performed as described above. Gene expression values, t-tests and p-values were calculated using Bio-Rad CFX Manager 3.1 software which uses the normalized expression calculation (ddCt) of Livak and Schmittgen (2001). Samples were normalized to the geometric mean of *efl1a* and *l13a* Cts and are shown as fold change differences from control embryos.

Primer sequences (5'–3'):

l13a F: AAGCAAGTGCTGTTGGGCCACA

l13a R: TGATCCACGGGAAGGGTTGGTGT

pgc1a F: GGCCAGCGAGCCAAACCAA

pgc1a R: TGGCTTTGTGAGGAGGCGTGG

peo1 F: AGCCTCGCTCACCTTCTCTGCT

peo1 R: TGCTGCTGTGACCCCTTGAC

opa1 F: GCCGGAAGTGTAGTTACCTG

opa1 R: AGGTGGTCTCTGTGGGTTGT

nd5 F: CCTCCCATCTTAACGCCTGAGCC

nd5 R: TCGTAGGTCTTGTGTCGGGGGC

sod2 F: TTCAGGGCTCAGGCTGG

sod2 R: ATGGCTTTAACATAGTCCGGT

cyp11a1 F: TGCCGATTTTCATCCCTTTCC

cyp11a1 R: AGAGCCGTGCTGATAGTGTC

nrf2 F: GACAAAATCGGCGACAAAAT

nrf2 R: TTAGGCCATGTCCACACGTA

ogg1 F: TCGGATTGTATTAGCCC

ogg1 R: AAACCCAAAGATGGAGG

hif1a F: GCGTAAGAGGAAGCTGAACG

hif1a R: TCCACACTGTGAAGCAAAGC

4.9. Respirometry

The XF-24 Extracellular Flux Analyzer (Seahorse Bioscience) was used to measure oxygen consumption rates (OCR) from 24, 48 and 72 hpf zebrafish embryos. This instrument measures OCR using solid state sensor probes that quantify the changing concentrations of dissolved oxygen and free protons in the micro-environment surrounding individual intact embryos. Zebrafish embryos were staged and placed with 700 μ L egg water in a 24 well islet plate, and kept in place with an islet capture screen. Cartridge sensors were calibrated and islet plates were loaded onto the instrument. After measuring basal respiration rates for all embryos, either FCCP, oligomycin, or sodium azide was delivered to a subset of the animals on each plate at concentrations previously established to produce the maximum change in respiration without inducing mortality (Rahn et al., 2013; Stackley et al., 2011). Total basal respiration was measured at two consecutive measurement cycles before the first injection, and these two measurements were then averaged. Drug response was determined by averaging the OCR at two consecutive measurement cycles, either at the maximum OCR (after FCCP injection), or the minimum OCR (after oligomycin or sodium azide injection).

4.10. Immunofluorescence and nuclear labeling

Protocols for processing whole mount zebrafish for immuno-fluorescence were adapted from Hutchinson and Eisen (2006) using buffer recipes from Westerfield (2007). Briefly, after fixation for 4 h at room temperature in 4% paraformaldehyde diluted in Fixation Buffer, the yolks were dissected away from the rest of the embryo, and the embryonic tissue was permeabilized in ddH₂O for 30 min (24 hpf embryos) to 2 h (for embryos 72 hpf or older). After rinsing in PBS and incubating in Blocking Solution for 1 h, the tissue was incubated for 1–4 days in primary antibodies diluted 1:50 in Blocking Solution with constant mixing at 4 °C. After 3 changes of Wash Solution, the tissue was incubated for 4 h at room temperature or up to 2 days at 4 °C in AlexaFluor 488, 543 or 568 secondary antibodies diluted 1:400 in Blocking Solution. In some cases the tissue was then incubated in 0.5 μ M Sytox Red (Invitrogen) for at least 24 h, as described in Picker et al. (1999) in order to label nuclei. After 3 changes of Wash Buffer, embryos were mounted in Slowfade Gold (Invitrogen) between coverslips using a hole-punched sticker as a spacer and sealed with Permount (Sigma). Dr. Bill Trevarrow developed the zn1/zn1 antibodies which were used together to label primary motor neurons in Trevarrow et al. (1990). The muscle pioneer cells were labeled by the 4d9 antibody developed by Dr. Corey Goodman (Patel et al., 1989). Cells from 3 or 4 somites per embryo were measured to derive the average number per

embryo. The 3a10 neurofilament antibody used to label the Mauthner reticulospinal neurons was developed by Drs. Thomas Jessell, Jane Dodd and Susan Brenner-Morton (Furley et al., 1990). We used the SV2 antibody developed by Dr. Kathleen Buckley to label synaptic vesicles (Buckley and Kelly, 1985). All primary antibodies used for immunofluorescence were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA.

4.11. Fish movement assay

Individual 72 hpf fish were transferred to wells of a 48 well tissue culture plate and placed into a Daniovision instrument (Noldus Information Technology) where a video was recorded for 27 min at 3.75 frames/s. All fish were alive at the end of the recording. The videos were processed using Virtual Dub (Avery Lee) and the FIJI image processing package of Image J to extract the 1 min interval images and calculate the differences between consecutive images. The resulting images were then thresholded to remove background levels, assembled into an image stack and projected to reveal the cumulative positions of each fish. Using the ROI Manager tool in ImageJ, the well areas were selected and the intensity per well was measured in order to derive the percent of each well masked by the cumulative positions of the fish.

4.12. In vivo cell labeling and imaging

We used 5-(and 6-)chloromethyl-2,7-dichlorodihydrofluorescein diacetate (CM-H2DCFDA; Invitrogen), a cell-permeable fluorescent indicator of ROS, in order to assess levels of oxidation in live control and DNP-treated embryos. Similar to other CM-H2DCFDA-labelling methods (Anichtchik et al., 2008; Walker et al., 2012), manually dechorionated control and CM-H2DCFDA-treated embryos were placed in 20 μ M CM-H2DCFDA for at least 1 h prior to 3×10 min washes in 0.3% Danieau saline. The in vivo marker for condensed chromatin, acridine orange (AO, Sigma), was used to assess levels of cell death in the eye as previously described (Shen and Raymond, 2004). Manually dechorionated embryos were incubated in 5 g/L AO for 10 min, followed by 3×10 min washes in 0.3% Danieau Saline.

Embryos were prepared for in vivo, two-photon imaging using methods similar to Walker et al. (2012). Briefly, embryos were anesthetized by submersion in 0.3 mM MS-222 anesthesia before being mounted in 0.5–1% low melting temperature agarose on a 35 mm petri dish with a glass bottom for imaging (MatTek Corporation). The mounted embryos were bathed in 0.3% Danieau Saline and 0.3 mM MS-222 during image acquisition.

4.13. Microscopy and image analysis

Single and two-photon images were acquired using a Zeiss LSM 510 META multiphoton system mounted on an Axioplan 2 microscope and equipped with 458, 488, 514, 543, 633 nm and a Verdi/Mira (Coherent) ti-sapphire laser. Images were acquired using Zeiss Plan-Neofluar 25x/0.8 NA, C Apochromat 40x/1.2 NA and LCI Plan-Neofluar 63x/1.3 NA objectives. Acquisition of fixed tissue immunofluorescence was conducted using sequential single photon excitation with optimal pinhole size calculated by the Zeiss software for

proper spatial resolution. Emission filters and dichroic mirrors were adjusted to minimize overlap in fluorescent signals. Multiphoton excitation was used for in vivo imaging experiments of AO staining (laser mode-locked at 836 nm) or CM-H2DCFDA (laser mode-locked at 905 nm).

For the CM-H2DCFDA experiments, we acquired 120 μm (4 μm z-steps interval) stacks through the heads of fish, using identical image acquisition settings and alternating between control and DNP-treated embryos. Using FIJI (Schindelin et al., 2012) an image processing package of ImageJ (Schneider et al., 2012), regions of interest were drawn around the head tissue and the mean fluorescence levels were measured from the maximum intensity projections of the confocal stacks. The data were normalized to the average control levels for each experiment and presented as means $\pm$ SEM. For the AO experiments, 120 μm (1 μm z-step interval) images were acquired through the eyes of control and DNP-treated embryos. Using FIJI and its ROI Manager and Point Selection tools, each AO-labeled cell body was marked and counted throughout the image stack. Measurements of somite lengths and neural tube, lens and eye diameters were conducted using drawing tools and ROI Manager FIJI plugins. All measurements of tail structures were limited to somites containing the yolk extension. The Simple Neurite Tracer plugin for FIJI (Longair et al., 2011) was used to measure the axon length from the exit point from the neural tube to its most distal branch tip of the caudal motor neurons.

4.14. Statistical analyses

Statistical analyses were performed using SPSS v21 (IBM), JMP10 (SAS), BioRad CFX Manager 3.1, or Excel (Microsoft). T-tests were used by the BioRad CFX Manager software to test gene expression and mtDNA fold change comparisons. All other comparisons between groups were conducted using a Wilcoxon rank sum (Mann–Whitney U) test. Within group comparisons were tested using the Wilcoxon Signed Rank test. Multiple comparison ANOVA results used the Steel-Dwass post-hoc test. Differences were considered statistically significant when $p < 0.05$. Data are plotted as means $\pm$ SEM with a significant difference indicated with an asterisk.

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Abbreviations

AO	acridine orange
CaP	caudal primary
DNP	2,4-dinitrophenol
FCCP	carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazine

GCL	ganglion cell layer
hpf	hours post-fertilization
INL	inner nuclear layer
L	lens
N	neural tube
N	notochord
PL	photoreceptor layer
RGC	retinal ganglion cell
mtDNA	mitochondrial DNA
OCR	oxygen consumption rate
OVL	otic vesicle length
OXPHOS	oxidative phosphorylation
ROS	reactive oxygen species
YE	yolk extension

References

- Agathocleous M, Harris WA. Metabolism in physiological cell proliferation and differentiation. *Trends Cell Biol.* 2013; 23:484–492. [PubMed: 23756093]
- Aizawa H, Bianco IH, Hamaoka T, Miyashita T, Uemura O, Concha ML, Russell C, Wilson SW, Okamoto H. Laterotopic representation of left–right information onto the dorso-ventral axis of a zebrafish midbrain target nucleus. *Curr. Biol.* 2005; 15:238–243. [PubMed: 15694307]
- Alexander C, Votruba M, Pesch UE, Thiselton DL, Mayer S, Moore A, Rodriguez M, Kellner U, Leo-Kottler B, Auburger G, Bhattacharya SS, Wissinger B. OPA1, encoding a dynamin-related GTPase, is mutated in autosomal dominant optic atrophy linked to chromosome 3q28. *Nat. Genet.* 2000; 26:211–215. [PubMed: 11017080]
- Anichtchik O, Diekmann H, Fleming A, Roach A, Goldsmith P, Rubinshtein DC. Loss of PINK1 function affects development and results in neurodegeneration in zebrafish. *J. Neurosci.* 2008; 28:8199–8207. [PubMed: 18701682]
- Antonenko YN, Khailova LS, Knorre DA, Markova OV, Rokitskaya TI, Ilyasova TM, Severina II, Kotova EA, Karavaeva YE, Prikhodko AS, Severin FF, Skulachev VP. Penetrating cations enhance uncoupling activity of anionic protonophores in mitochondria. *PLoS One.* 2013; 8:e61902. [PubMed: 23626747]
- Balaban RS, Nemoto S, Finkel T. Mitochondria, oxidants, and aging. *Cell.* 2005; 120:483–495. [PubMed: 15734681]
- Beattie CE, Carrel TL, McWhorter ML. Fishing for a mechanism: using zebrafish to understand spinal muscular atrophy. *J. Child Neurol.* 2007; 22:995–1003. [PubMed: 17761655]
- Bender A, Krishnan KJ, Morris CM, Taylor GA, Reeve AK, Perry RH, Jaros E, Hersheson JS, Betts J, Klopstock T, Taylor RW, Turnbull DM. High levels of mitochondrial DNA deletions in substantia nigra neurons in aging and Parkinson disease. *Nat. Genet.* 2006; 38:515–517. [PubMed: 16604074]
- Bishop T, Brand MD. Processes contributing to metabolic depression in hepatopancreas cells from the snail *Helix aspersa*. *J. Exp. Biol.* 2000; 203:3603–3612. [PubMed: 11060221]
- Brand MD. Uncoupling to survive? The role of mitochondrial inefficiency in ageing. *Exp. Gerontol.* 2000; 35:811–820. [PubMed: 11053672]

- Brand MD, Nicholls DG. Assessing mitochondrial dysfunction in cells. *Biochem. J.* 2011; 435:297–312. [PubMed: 21726199]
- Brandt RB, Siegel SA, Waters MG, Bloch MH. Spectrophotometric assay for D-(–)-lactate in plasma. *Anal. Biochem.* 1980; 102:39–46. [PubMed: 7356162]
- Brocato J, Chervona Y, Costa M. Molecular responses to hypoxia-inducible factor 1alpha and beyond. *Mol. Pharmacol.* 2014; 85:651–657. [PubMed: 24569087]
- Buckler KJ, Vaughnan-Jones RD. Effects of mitochondrial uncouplers on intracellular calcium, pH and membrane potential in rat carotid body type I cells. *J. Physiol.* 1998;513. [PubMed: 9706000]
- Buckley K, Kelly RB. Identification of a transmembrane glycoprotein specific for secretory vesicles of neural and endocrine cells. *J. Cell Biol.* 1985; 100:1284–1294. [PubMed: 2579958]
- Caldeira da Silva CC, Cerqueira FM, Barbosa LF, Medeiros MH, Kowaltowski AJ. Mild mitochondrial uncoupling in mice affects energy metabolism, redox balance and longevity. *Aging Cell.* 2008; 7:552–560. [PubMed: 18505478]
- Carelli V, La Morgia C, Valentino ML, Barboni P, Ross-Cisneros FN, Sadun AA. Retinal ganglion cell neurodegeneration in mitochondrial inherited disorders. *Biochim. Biophys. Acta.* 2009; 1787:518–528. [PubMed: 19268652]
- Celotto AM, Liu Z, Vandemark AP, Palladino MJ. A novel *Drosophila* SOD2 mutant demonstrates a role for mitochondrial ROS in neurodevelopment and disease. *Brain Behav.* 2012; 2:424–434. [PubMed: 22950046]
- Chinnery PF, Hudson G. Mitochondrial genetics. *Br. Med. Bull.* 2013; 106:135–159. [PubMed: 23704099]
- Cole LK, Ross LS. Apoptosis in the developing zebrafish embryo. *Dev. Biol.* 2001; 240:123–142. [PubMed: 11784051]
- da Costa RF, Martinez AM, Ferreira ST. 2,4-Dinitrophenol blocks neuro-degeneration and preserves sciatic nerve function after trauma. *J. Neurotrauma.* 2010; 27:829–841. [PubMed: 20143955]
- Dechant R, Binda M, Lee SS, Pelet S, Winderickx J, Peter M. Cytosolic pH is a second messenger for glucose and regulates the PKA pathway through V-ATPase. *EMBO J.* 2010; 29:2515–2526. [PubMed: 20581803]
- Delettre C, Lenaers G, Griffoin JM, Gigarel N, Lorenzo C, Belenguer P, Pelloquin L, Grosgeorge J, Turc-Carel C, Perret E, Astarie-Dequeker C, Lasquellerc L, Arnaud B, Ducommun B, Kaplan J, Hamel CP. Nuclear gene OPA1, encoding a mitochondrial dynamin-related protein, is mutated in dominant optic atrophy. *Nat. Genet.* 2000; 26:207–210. [PubMed: 11017079]
- Dennery PA. Effects of oxidative stress on embryonic development. *Birth defects research. Part C. Embryo Today: Rev.* 2007; 81:155–162.
- Diering GH, Numata M. Endosomal pH in neuronal signaling and synaptic transmission: role of Na(+)/H(+) exchanger NHE5. *Front. Physiol.* 2014; 4:412. [PubMed: 24454292]
- DiMauro S, Schon EA. Mitochondrial disorders in the nervous system. *Annu. Rev. Neurosci.* 2008; 31:91–123. [PubMed: 18333761]
- Erecinska M, Cherian S, Silver IA. Energy metabolism in mammalian brain during development. *Prog. Neurobiol.* 2004; 73:397–445. [PubMed: 15313334]
- Facucho-Oliveira St JM, John JC. The relationship between pluripotency and mitochondrial DNA proliferation during early embryo development and embryonic stem cell differentiation. *Stem Cell Rev.* 2009; 5:140–158. [PubMed: 19521804]
- Fernandez-Vizarra E, Enriquez JA, Perez-Martos A, Montoya J, Fernandez-Silva P. Tissue-specific differences in mitochondrial activity and biogenesis. *Mitochondrion.* 2011; 11:207–213. [PubMed: 20933104]
- Finkel T. Signal transduction by reactive oxygen species. *J Cell Biol.* 2011; 194:7–15. [PubMed: 21746850]
- Flanagan-Steet HR, Steet R. “Casting” light on the role of glycosylation during embryonic development: insights from zebrafish. *Glycoconj. J.* 2013; 30:33–40. [PubMed: 22638861]
- Flynn JM, Melov S. SOD2 in mitochondrial dysfunction and neurodegeneration. *Free Rad. Biol. Med.* 2013; 62:4–12. [PubMed: 23727323]

- Furley AJ, Morton SB, Manalo D, Karagogeos D, Dodd J, Jessell TM. The axonal glycoprotein TAG-1 is an immunoglobulin superfamily member with neurite outgrowth-promoting activity. *Cell*. 1990; 61:157–170. [PubMed: 2317872]
- Furutani-Seiki M, Jiang YJ, Brand M, Heisenberg CP, Houart C, Beuchle D, van Eeden FJ, Granato M, Haffter P, Hammerschmidt M, Kane DA, Kelsh RN, Mullins MC, Odenthal J, Nusslein-Volhard C. Neural degeneration mutants in the zebrafish, *Danio rerio*. *Development*. 1996; 123:229–239. [PubMed: 9007243]
- Haas RH, Parikh S, Falk MJ, Saneto RP, Wolf NI, Darin N, Cohen BH. Mitochondrial disease: a practical approach for primary care physicians. *Pediatrics*. 2007; 120:1326–1333. [PubMed: 18055683]
- Haas RH, Parikh S, Falk MJ, Saneto RP, Wolf NI, Darin N, Wong LJ, Cohen BH, Naviaux RK. The in-depth evaluation of suspected mitochondrial disease. *Mol. Genet. Metab*. 2008; 94:16–37. [PubMed: 18243024]
- Harper M-E, Brand MD. The quantitative contributions of mitochondrial proton leak and ATP turnover reactions to the changed respiration rates of hepatocytes from rats of different thyroid status. *J. Biol. Chem*. 1993; 268(20):14850–14860. <http://www.ncbi.nlm.nih.gov/pubmed/8392060>. [PubMed: 8392060]
- Harris, MO.; Corcoran, JJ. Agency for Toxic Substances and Disease Registry for the U.S. Department of Health and Human Services; Atlanta, GA.: 1995. Toxicological Profile for Dinitrophenols..
- Hayes JD, Dinkova-Kostova AT. The Nrf2 regulatory network provides an interface between redox and intermediary metabolism. *Trends Biochem. Sci*. 2014; 39:199–218. [PubMed: 24647116]
- Hock MB, Kralli A. Transcriptional control of mitochondrial biogenesis and function. *Annu. Rev. Physiol*. 2009; 71:177–203. [PubMed: 19575678]
- Hudman D, Rainbow RD, Lawrence CL, Standen NB. The origin of calcium overload in rat cardiac myocytes following metabolic inhibition with 2,5-dinitrophenol. *J. Mol. Cell Cardiol*. 2002; 34:859–871. [PubMed: 12099724]
- Hutchinson SA, Eisen JS. Islet1 and Islet2 have equivalent abilities to promote motoneuron formation and to specify motoneuron subtype identity. *Development*. 2006; 133:2137–2147. [PubMed: 16672347]
- Jastroch M, Divakaruni AS, Mookerjee S, Treberg JR, Brand MD. Mitochondrial proton and electron leaks. *Essays Biochem*. 2010; 47:53–67. [PubMed: 20533900]
- Kageyama Y, Zhang Z, Sesaki H. Mitochondrial division: molecular machinery and physiological functions. *Curr. Opin. Cell Biol*. 2011; 23:427–434. [PubMed: 21565481]
- Karlsson M, Kurz T, Brunk UT, Nilsson SE, Frennsson CI. What does the commonly used DCF test for oxidative stress really show? *Biochem. J*. 2010; 428:183–190. [PubMed: 20331437]
- Katsetos CD, Koutzaki S, Melvin JJ. Mitochondrial dysfunction in neuro-muscular disorders. *Sem. Pediatr. Neurol*. 2013; 20:202–215.
- Kay JN, Roeser T, Mumm JS, Godinho L, Mrejeru A, Wong RO, Baier H. Transient requirement for ganglion cells during assembly of retinal synaptic layers. *Development*. 2004; 131:1331–1342. [PubMed: 14973290]
- Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. Stages of embryonic development of the zebrafish. *Dev. Dyn*. 1995; 203:253–310. [PubMed: 8589427]
- Kluver N, Yang L, Busch W, Scheffler K, Renner P, Strahle U, Scholz S. Transcriptional response of zebrafish embryos exposed to neurotoxic compounds reveals a muscle activity dependent hspb11 expression. *PLoS One*. 2011; 6:e29063. [PubMed: 22205996]
- Koenig MK. Presentation and diagnosis of mitochondrial disorders in children. *Pediatr. Neurol*. 2008; 38:305–313. [PubMed: 18410845]
- Korde AS, Pettigrew LC, Craddock SD, Maragos WF. The mitochondrial uncoupler 2,4-dinitrophenol attenuates tissue damage and improves mitochondrial homeostasis following transient focal cerebral ischemia. *J. Neurochem*. 2005; 94:1676–1684. [PubMed: 16045446]
- Korn H, Faber DS. The Mauthner cell half a century later: a neurobiological model for decision-making? *Neuron*. 2005; 47:13–28. [PubMed: 15996545]
- Lee JM, Li J, Johnson DA, Stein TD, Kraft AD, Calkins MJ, Jakel RJ, Johnson JA. Nrf2, a multi-organ protector? *FASEB J*. 2005; 19:1061–1066. [PubMed: 15985529]

- Lennie P. The cost of cortical computation. *Curr. Biol.* 2003; 13:493–497. [PubMed: 12646132]
- Levine RL, Wehr N, Williams JA, Stadtman ER, Shacter E. Determination of carbonyl groups in oxidized proteins. *Methods Mol. Biol.* 2000; 99:15–24. [PubMed: 10909073]
- Li Z, Hu M, Ochocinska MJ, Joseph NM, Easter SS Jr. Modulation of cell proliferation in the embryonic retina of zebrafish (*Danio rerio*). *Dev. Dyn.* 2000; 219:391–401. [PubMed: 11066095]
- Liesa M, Shirihai OS. Mitochondrial dynamics in the regulation of nutrient utilization and energy expenditure. *Cell Metab.* 2013; 17:491–506. [PubMed: 23562075]
- Lin J, Handschin C, Spiegelman BM. Metabolic control through the PGC-1 family of transcription coactivators. *Cell Metab.* 2005; 1:361–370. [PubMed: 16054085]
- Liu Y, Fiskum G, Schubert D. Generation of reactive oxygen species by the mitochondrial electron transport chain. *J. Neurochem.* 2002; 80:780–787. [PubMed: 11948241]
- Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods.* 2001; 25:402–408. [PubMed: 11846609]
- Longair MH, Baker DA, Armstrong JD. Simple Neurite Tracer: open source software for reconstruction, visualization and analysis of neuronal processes. *Bioinformatics.* 2011; 27:2453–2454. [PubMed: 21727141]
- Lou PH, Hansen BS, Olsen PH, Tullin S, Murphy MP, Brand MD. Mitochondrial uncouplers with an extraordinary dynamic range. *Biochem. J.* 2007; 407:129–140. [PubMed: 17608618]
- Luo S, Wehr NB. Protein carbonylation: avoiding pitfalls in the 2,4-dinitrophenylhydrazine assay. *Redox Rep.: Commun. Free Rad. Res.* 2009; 14:159–166.
- Maresca A, la Morgia C, Caporali L, Valentino ML, Carelli V. The optic nerve: a “mito-window” on mitochondrial neurodegeneration. *Mol. Cell Neurosci.* 2013; 55:62–76. [PubMed: 22960139]
- Marit JS, Weber LP. Acute exposure to 2,4-dinitrophenol alters zebrafish swimming performance and whole body triglyceride levels. *Comparative biochemistry and physiology. Toxicol. Pharmacol.: CBP.* 2011; 154:14–18.
- Mattiasson G, Shamloo M, Gido G, Mathi K, Tomasevic G, Yi S, Warden CH, Castilho RF, Melcher T, Gonzalez-Zulueta M, Nikolich K, Wieloch T. Uncoupling protein-2 prevents neuronal death and diminishes brain dysfunction after stroke and brain trauma. *Nat. Med.* 2003; 9:1062–1068. [PubMed: 12858170]
- Maynard S, Keijzers G, Gram M, Desler C, Bendix L, Budtz-Jorgensen E, Molbo D, Croteau DL, Osler M, Stevnsner T, Rasmussen LJ, Dela F, Avlund K, Bohr VA. Relationships between human vitality and mitochondrial respiratory parameters, reactive oxygen species production and dNTP levels in peripheral blood mononuclear cells. *Aging.* 2013; 5:850–864. [PubMed: 24304678]
- McGown A, McDearmid JR, Panagiotaki N, Tong H, Al Mashhadi S, Redhead N, Lyon AN, Beattie CE, Shaw PJ, Ramesh TM. Early interneuron dysfunction in ALS: insights from a mutant *sod1* zebrafish model. *Ann. Neurol.* 2013; 73:246–258. [PubMed: 23281025]
- Mellman I. The importance of being acid: the role of acidification in intracellular membrane traffic. *J. Exp. Biol.* 1992; 172:39–45. [PubMed: 1491231]
- Menezes MP, Ouvrier RA. Peripheral neuropathy associated with mitochondrial disease in children. *Dev. Med. Child Neurol.* 2012; 54:407–414. [PubMed: 22435634]
- Meyers PZ, Eisen JS, Westerfield M. Development and axonal outgrowth of identified motoneurons in the zebrafish. *J. Neurosci.* 1986; 6:2278–2289. [PubMed: 3746410]
- Muller JS, Jepson CD, Laval SH, Bushby K, Straub V, Lochmuller H. Dok-7 promotes slow muscle integrity as well as neuromuscular junction formation in a zebrafish model of congenital myasthenic syndromes. *Hum. Mol. Genet.* 2010; 19:1726–1740. [PubMed: 20147321]
- Mumm JS, Godinho L, Morgan JL, Oakley DM, Schroeter EH, Wong RO. Laminar circuit formation in the vertebrate retina. *Prog. Brain Res.* 2005; 147:155–169. [PubMed: 15581704]
- Mumm JS, Williams PR, Godinho L, Koerber A, Pittman AJ, Roeser T, Chien CB, Baier H, Wong RO. In vivo imaging reveals dendritic targeting of laminated afferents by zebrafish retinal ganglion cells. *Neuron.* 2006; 52:609–621. [PubMed: 17114046]
- Munnich A, Rotig A, Chretien D, Cormier V, Bourgeron T, Bonnefont JP, Saudubray JM, Rustin P. Clinical presentation of mitochondrial disorders in childhood. *J. Inherit. Metab. Dis.* 1996; 19:521–527. [PubMed: 8884575]

- Murphy MP. How mitochondria produce reactive oxygen species. *Biochem. J.* 2009; 417:1–13. [PubMed: 19061483]
- Nadler JE. Peripheral neuritis caused by prolonged use of dinitrophenol. *J. Am. Med. Assoc.* 1935; 105:12–13.
- Orlowski J, Grinstein S. Emerging roles of alkali cation/proton exchangers in organellar homeostasis. *Curr. Opin. Cell Biol.* 2007; 19:483–492. [PubMed: 17646094]
- Pandya JD, Pauly JR, Nukala VN, Sebastian AH, Day KM, Korde AS, Maragos WF, Hall ED, Sullivan PG. Post-injury administration of mitochondrial uncouplers increases tissue sparing and improves behavioral outcome following traumatic brain injury in rodents. *J. Neurotrauma.* 2007; 24:798–811. [PubMed: 17518535]
- Patel NH, Martin-Blanco E, Coleman KG, Poole SJ, Ellis MC, Kornberg TB, Goodman CS. Expression of engrailed proteins in arthropods, annelids, and chordates. *Cell.* 1989; 58:955–968. [PubMed: 2570637]
- Phillips L, Singer MA. Peripheral neuropathy due to dinitrophenol used for weight loss: something old, something new. *Neurology.* 2013; 80:773–774. [PubMed: 23365059]
- Picker A, Brennan C, Reifers F, Clarke JD, Holder N, Brand M. Requirement for the zebrafish mid-hindbrain boundary in midbrain polarisation, mapping and confinement of the retinotectal projection. *Development.* 1999; 126:2967–2978. [PubMed: 10357940]
- Pinho BR, Santos MM, Fonseca-Silva A, Valentao P, Andrade PB, Oliveira JM. How mitochondrial dysfunction affects zebrafish development and cardiovascular function: an in vivo model for testing mitochondria-targeted drugs. *Br. J. Pharmacol.* 2013; 169:1072–1090. [PubMed: 23758163]
- PubChem. qHTS assay for small molecule agonists of the antioxidant response element (ARE) signaling pathway: Summary. NCBI. , editor. *BioAssay*; 2014. p. AID 743219
- Rahn JJ, Stackley KD, Chan SS. Opa1 is required for proper mitochondrial metabolism in early development. *PLoS One.* 2013; 8:e59218. [PubMed: 23516612]
- Raimundo N. Mitochondrial pathology: stress signals from the energy factory. *Trends Mol. Med.* 2014; 20:282–292. [PubMed: 24508276]
- Reis A, Hermanson O. The DNA glycosylases OGG1 and NEIL3 influence differentiation potential, proliferation, and senescence-associated signs in neural stem cells. *Biochem. Biophys. Res. Commun.* 2012; 423:621–626. [PubMed: 22564741]
- Sambrook, J.; Russell, DW. *Molecular Cloning, A Laboratory Manual.* Cold Spring Harbor Laboratory Press; Cold Spring Harbor, NY.: 2001.
- Scarpulla RC. Transcriptional paradigms in mammalian mitochondrial biogenesis and function. *Physiol. Rev.* 2008; 88:611–638. [PubMed: 18391175]
- Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez JY, White DJ, Hartenstein V, Eliceiri K, Tomancak P, Cardona A. Fiji: an open-source platform for biological-image analysis. *Nat. Methods.* 2012; 9:676–682. [PubMed: 22743772]
- Schmitt EA, Dowling JE. Early eye morphogenesis in the zebrafish, *Brachydanio rerio*. *J. Comp. Neurol.* 1994; 344:532–542. [PubMed: 7929890]
- Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods.* 2012; 9:671–675. [PubMed: 22930834]
- Shen YC, Raymond PA. Zebrafish cone-rod (crx) homeobox gene promotes retinogenesis. *Dev. Biol.* 2004; 269:237–251. [PubMed: 15081370]
- Shivdasani RA, Orkin SH. Erythropoiesis and globin gene expression in mice lacking the transcription factor NF-E2. *Proc. Natl. Acad. Sci.* 1995; 92(19):8690–8694. [PubMed: 7567998]
- Singh B, Chatterjee A, Ronghe AM, Bhat NK, Bhat HK. Antioxidant-mediated up-regulation of OGG1 via NRF2 induction is associated with inhibition of oxidative DNA damage in estrogen-induced breast cancer. *BMC Cancer.* 2013; 13:253. [PubMed: 23697596]
- Spelbrink JN, Li FY, Tiranti V, Nikali K, Yuan QP, Tariq M, Wanrooij S, Garrido N, Comi G, Morandi L, Santoro L, Toscano A, Fabrizi GM, Somer H, Croxen R, Beeson D, Poulton J, Suomalainen A, Jacobs HT, Zeviani M, Larsson C. Human mitochondrial DNA deletions

associated with mutations in the gene encoding Twinkle, a phage T7 gene 4-like protein localized in mitochondria. *Nat. Genet.* 2001; 28:223–231. [PubMed: 11431692]

- Stackley KD, Beeson CC, Rahn JJ, Chan SS. Bioenergetic profiling of zebrafish embryonic development. *PLoS One.* 2011; 6:e25652. [PubMed: 21980518]
- Steele SL, Prykhodzhiy SV, Berman JN. Zebrafish as a model system for mitochondrial biology and diseases. *Transl. Res.: J. Lab. Clin. Med.* 2014; 163:79–98.
- Trachootham D, Lu W, Ogasawara MA, Nilsa RD, Huang P. Redox regulation of cell survival. *Antioxid. Redox Signal.* 2008; 10:1343–1374. [PubMed: 18522489]
- Trevarrow B, Marks DL, Kimmel CB. Organization of hindbrain segments in the zebrafish embryo. *Neuron.* 1990; 4:669–679. [PubMed: 2344406]
- Trounce I. Genetic control of oxidative phosphorylation and experimental models of defects. *Hum. Reprod.* 2000; 15(Suppl. 2):S18–S27.
- Tsatmali M, Walcott EC, Crossin KL. Newborn neurons acquire high levels of reactive oxygen species and increased mitochondrial proteins upon differentiation from progenitors. *Brain Res.* 2005; 1040:137–150. [PubMed: 15804435]
- Turrens JF. Mitochondrial formation of reactive oxygen species. *J. Physiol.* 2003; 552:335–344. [PubMed: 14561818]
- Tyynismaa H, Sembongi H, Bokori-Brown M, Granycome C, Ashley N, Poulton J, Jalanko A, Spelbrink JN, Holt IJ, Suomalainen A. Twinkle helicase is essential for mtDNA maintenance and regulates mtDNA copy number. *Hum. Mol. Genet.* 2004; 13:3219–3227. [PubMed: 15509589]
- Valero T, Moschopoulou G, Mayor-Lopez L, Kintzios S. Moderate super-oxide production is an early promoter of mitochondrial biogenesis in differentiating N2a neuroblastoma cells. *Neurochem. Int.* 2012; 61:1333–1343. [PubMed: 23022608]
- Ventura-Clapier R, Garnier A, Veksler V. Transcriptional control of mitochondrial biogenesis: the central role of PGC-1alpha. *Cardiovasc. Res.* 2008; 79:208–217. [PubMed: 18430751]
- Walker SL, Ariga J, Mathias JR, Coothankandaswamy V, Xie X, Distel M, Koster RW, Parsons MJ, Bhalla KN, Saxena MT, Mumm JS. Automated reporter quantification in vivo: high-throughput screening method for reporter-based assays in zebrafish. *PLoS One.* 2012; 7:e29916. [PubMed: 22238673]
- Wang P, Powell SR. Decreased sensitivity associated with an altered formulation of a commercially available kit for detection of protein carbonyls. *Free Rad. Biol. Med.* 2010; 49:119–121. [PubMed: 20230891]
- Wei Y, Gong J, Thimmulappa RK, Kosmider B, Biswal S, Duh EJ. Nrf2 acts cell-autonomously in endothelium to regulate tip cell formation and vascular branching. *Proc. Natl. Acad. Sci. USA.* 2013; 110:E3910–3918. [PubMed: 24062466]
- Weil M, Scholz S, Zimmer M, Sacher F, Duis K. Gene expression analysis in zebrafish embryos: a potential approach to predict effect concentrations in the fish early life stage test. *Environ. Toxicol. Chem.* 2009; 28:1970–1978. [PubMed: 19402758]
- Westerfield, M. *The Zebrafish Book. A Guide for the Laboratory use of Zebrafish (Danio rerio).* 5th ed.. University of Oregon Press; Eugene, OR.: 2007.
- Williams LM, Timme-Laragy AR, Goldstone JV, McArthur AG, Stegeman JJ, Smolowitz RM, Hahn ME. Developmental expression of the Nfe2-related factor (Nrf) transcription factor family in the zebrafish, *Danio rerio*. *PLoS One.* 2013; 8:e79574. [PubMed: 24298298]
- Yan L, Zhou Y, Yu S, Ji G, Wang L, Liu W, Gu A. 8-Oxoguanine DNA glycosylase 1 (ogg1) maintains the function of cardiac progenitor cells during heart formation in zebrafish. *Exp. Cell Res.* 2013; 319:2954–2963. [PubMed: 23892003]
- Yin C, Solnica-Krezel L. Convergence and extension movements mediate the specification and fate maintenance of zebrafish slow muscle precursors. *Dev. Biol.* 2007; 304:141–155. [PubMed: 17239849]
- Yu-Wai-Man P, Griffiths PG, Chinnery PF. Mitochondrial optic neuropathies – disease mechanisms and therapeutic strategies. *Prog. Retin Eye Res.* 2011; 30:81–114. [PubMed: 21112411]
- Zon LI, Peterson RT. In vivo drug discovery in the zebrafish. *Nat. Rev.: Drug Discov.* 2005; 4:35–44. [PubMed: 15688071]

Zsurka G, Kunz WS. Mitochondrial involvement in neurodegenerative diseases. IUBMB Life. 2013; 65:263–272. [PubMed: 23341346]

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Fig. 1.

DNP induces mitochondrial dysfunction, inhibits mitochondrial ATP production and prevents normal developmental increases in mitochondrial output. In vivo respiration (oxygen consumption) rates were measured from intact zebrafish embryos. (A) At 6 hpf, basal respiration was significantly increased in DNP-treated embryos compared to control fish indicating DNP uncoupled the mitochondria and compromised OXPHOS. $n=9$ and 8. (B) At 24 hpf, basal respiration of DNP treated embryos is not significantly different from control levels. The normal developmental increases basal respiration seen in the control animals are inhibited by DNP exposure. (C) Maximal respiration is decreased in DNP-exposed embryos at the later 48 and 72 hpf time points, but is not changed at 24 hpf. (D) Compared to controls, the ATP linked (oligomycin-sensitive) respiration of DNP-treated embryos is significantly decreased at all time points. (E) Non-mitochondrial respiration (azide-insensitive) respiration is decreased in DNP-exposed embryos at 24 and 72 hpf. (F) Proton leak in DNP-treated embryos is significantly higher than controls only at 24 hpf, $n=27-38$ embryos per time point. *Indicates significant ($p<0.05$) difference from control values or significant difference between time points.

Fig. 2.

After an initial compensatory increase in glycolysis, chronic DNP exposure lowers ATP levels and glycolytic output. (A) Embryos exposed to DNP have lower than control ATP levels at 48 and 72 hpf, but are not different at 24 hpf, $n=7$ replicates of 10 embryos. (B) DNP exposure produced a significant increase in whole embryo lactate levels at 24 hpf, indicating that glycolysis is induced. At 48 and 72 hpf, DNP-exposure significantly inhibited lactate production compared to control embryos. $n=5-6$ replicates each containing 10 embryos. * Indicates significant difference ($p<0.05$) from control values.

Fig. 3.

Levels of protein oxidation are lower in DNP-treated embryos. (A) Densitometry of levels of carbonylated protein normalized to B-actin reveals that DNP treated embryos have lower protein oxidation at 24 and 48 hpf. $n=3-7$ replicates of 10 animals. $*p<0.05$. (B-F) 2-photon image projections of H₂DCFDA fluorescence from control (B, C) and DNP-treated (D, E) embryos. Scale bar=300 μ m. (F) DNP-treated embryos had significantly lower levels of the H₂DCFDA fluorescent product, indicating lower levels of protein oxidation than control embryos at all time points. $n=12-30$ embryos/time point. * Indicates significant difference ($p<0.05$) from control values.

Fig. 4.

DNP exposure increases mtDNA abundance and induces changes in mitochondrial gene expression. (A) DNP treatment increases mtDNA at 12 hpf which is sustained until 96 hpf, indicating that mitochondrial biogenesis is induced. (B) With DNP treatment, the expression of mitochondrial transcription factors/activators *nrf2* and *pgc1a* peak at 6 hpf. *pgc1a* remains near control levels and *nrf2* expression increases at the 48 hpf time point. *hif1a* expression is equal to control levels except at 72 hpf when it is slightly elevated. (C) After the peak expression of mitochondrial maintenance genes at 6 hpf, all fall equal to or less

than control levels for the duration of embryonic development with the exception of *peo1* and *nd5* which show a transient increase at 48 hpf. (D) While *sod2* levels are significantly decreased, indicating lower ROS levels, the high *ogg1* expression at 48 and 72 hpf suggests DNA repair mechanisms are induced. qPCR gene expression values are normalized to the geometric mean of *ef1a*, *l13a* and/or *B-actin* expression and given as a fold-change from control levels. *n*=8–10 replicates of 20 pooled embryos. * Indicates a significant difference ($p<0.05$) in expression from control levels. Details are reported in Table 1.

Fig. 5.

DNP causes specific developmental abnormalities, decreases growth rates, and induces death at 72 hpf. Compared to control embryos (A–D), DNP-treated embryos (E, F) are smaller with underdeveloped eyes and indistinct brain regions (arrow, E). By 48 hpf, DNP-treated embryos (F) have smaller eyes and heads as well as pericardial edema (arrow, F) compared to control animals (B). At 72 hpf, pericardial edema was common among the DNP-treated embryos (arrow, G). Compared to control 72 hpf embryos (C and D), DNP embryos (G, H) also maintained their smaller head and eyes as well as shorter total body length (C and D versus G and H). Panels A–C, EG; scale bars=200 μm. Panels D, H: scale bar=500 μm. (I) The length of the embryos was significantly decreased by exposure to DNP, $n=27-30$, $p<0.01$. (J) While the otic vesicle length ratio (OVL) of control embryos decreases with age, DNP-treated embryos had significantly longer OVL measurements, $n=27-30$; $*p<0.002$. (K) The average width of the trunk somites containing the yolk extension was not different at 24 hpf, but DNP exposure significantly inhibited somite growth at 48 and 72 hpf, $n=14-25$, $p<0.05$. (L) DNP-induced mitochondrial dysfunction caused a significant decrease in the survival of 72 hpf embryos, $n=14$ clutches of ~300 embryos. * Indicates significant difference ($p<0.05$) from control values.

Fig. 6.

DNP treatment significantly inhibits the movement of 72 hpf fish. (A) Images of individual fish within wells of a 48 well plate were acquired at 1 min intervals for 27 min and the differences between consecutive images are shown as black pixels in each well, indicating that the fish had moved. Arrows indicate starting positions of fish that changed positions. All control fish (rows 1–2) changed positions at least once, but the majority of the DNP-treated fish (rows 3–6) did not move from their starting position as indicated as a completely white well. (B) The average percent of the well masked by the cumulative positions of the control fish was significantly greater than that of the DNP-treated fish indicating that DNP exposure inhibits the movement of zebrafish embryos. * Indicates significant difference ($p < 0.05$) from control levels.

Fig. 7.

DNP exposure does not alter Mauthner neurons or number of muscle pioneer cells. (A) Zebrafish diagram indicating somites above yolk extension and positions of images in B and C for the measurements in D. (B, C). Projections of confocal z-stacks taken of the engrailed-labeled muscle pioneer cells (green) from control (B) and DNP-treated (C) embryos at 24 hpf with Sytox-Red labeled nuclei (magenta) revealing the chevron-shaped somite boundaries. Scale=50 μm. (D) The average number of muscle pioneers per somite was not different between control and DNP-treated embryos, $n=10$ control and 6 DNP-treated embryos. $p=0.9566$. (E) Neural tube dorsal–ventral diameter of DNP-treated embryos does not differ significantly from the control diameter over embryonic development, $n=8–16$ control and $n=7–14$ DNP-treated embryos per time point. (F) Diagram of zebrafish head indicating area of confocal images shown in G and H. (G, H) Projections of confocal stacks reveal that DNP-treated embryos (H) have Mauthner neurons, with midline crossing axon projections, similar to control animals (G). Scale=50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 8.

DNP-treated zebrafish embryos fail to develop full motor neuron axon arbors. (A–D) Z-projections of confocal stacks taken through fixed whole mount 48 hpf embryos processed to reveal motor neurons (zn1/znf1; green) and the nuclear marker, SYTOX-red (magenta). The myotomes containing the yolk extension in the trunk of a control zebrafish embryo (A, B) and DNP-treated embryo (C, D) are shown. Images are oriented with caudal to the right and dorsal is up. The boxed regions in A and C are rotated 90° and projected in B and D, revealing the limited complexity of the axonal arbors in the DNP-treated embryo. The arrows in A and C indicate the tip of the caudal primary (CaP) motor neuron axons, which are shorter in the DNP-treated embryos. Scale bar=100 μm. E) The primary axon of the CaP motor neurons (diagrammed in B and D) were reconstructed from the 3D confocal stacks. The average CaP motor neuron axon lengths at 24, 48 and 72 hpf DNP-treated embryos are significantly shorter than the axons of control embryos, $n=5-10$. * Indicates significant difference ($p<0.05$) from control levels. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 9.

DNP exposure significantly reduces the size of the eye and induces acridine orange-labeled dying cells. (A) At 24 hpf, control and DNP-treated embryo eye size does not differ, but both retina and lens diameters of DNP-treated embryos are significantly smaller at 48 and 72 hpf, $n=19-39$, $*p<0.05$. (B–H) Acridine Orange staining reveals higher numbers of dying cells at 48 and 72 hpf in the eyes of DNP-treated embryos. The AO-labeled puncta from 7 to 18 eyes of control and 10–13 eyes of DNP-treated embryos were counted from the z-stacks acquired from 24, 48 and 72 hpf embryos. At 24 hpf, there is a slight but significant

decrease in AO-labeled apoptotic cells in the lenses of DNP-treated embryos. At 48 and 72 hpf, DNP-treated embryos have significantly greater levels of cell death in both their retina and lens *Indicates significant difference ($p<0.05$) from control levels. (C–H) Projected images of 2-photon stacks acquired through the eyes of control (C–E) and DNP-treated (F–H) embryos labeled AO. The arrows in E point to examples of surface pigment cells that develop in older animals and were not included in the analysis. Scale bar=100 μm .

Fig. 10.

DNP treated embryos lack retinal laminae and have less defined optic nerves. (A–D) An optical plane from control (A–B) and DNP-treated 72 hpf zebrafish eyes (C, D); boxes in A and C are enlarged in B and D. (A, B) In control animals, synaptic vesicle glycoprotein 2, sv2 (green) labels outer and inner plexiform layers (OPL and IPL). The nuclear marker, SYTOX Red (magenta) labels the photoreceptor layer (PL), the inner nuclear layer (INL) and ganglion cell layer (GCL). (C, D) DNP treated fish have indistinct sv2-labeled optic nerves (ON) compared to control embryos (arrows in B and D) and lack retinal layers. Arrows (C) and asterisk in D indicate the faint IPL. Scale bars=50μm. (E–L) Brightfield (E, G, I, K) and maximum projections of multiphoton 20 μm z-stacks of retina Brn3a:GFP expression (F, H, J, and L) from control (E, F) and DNP-treated (G–L) 72 hpf embryos. Arrows indicate Brn3a:GFP-labeled retinal ganglion cells. DNP-treated embryos have fewer retinal ganglion cells (G and I) and have disorganized GCL with GFP-positive cells found outside the GCL (asterisk, J, L). The nonspecific signal around the perimeter of the eye (J, L) is due to the pigmented epithelium. Scale bar=100μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1

mtDNA and mitochondrial-related RNA expression levels from DNP-treated embryos.

Symbol	Function	6 hpf	12 hpf	24 hpf	48 hpf	72 hpf	96 hpf
mtDNA	Estimates abundance of mitochondria	0.91 ± 0.17	1.20 ± 0.15	1.77 ± 0.37	1.35 ± 0.07	1.45 ± 0.04	0.96 ± 0.04
Mitochondrial DNA		(<i>p</i> = 0.26) <i>N</i> = 3	(<i>p</i> = 0.024) <i>N</i> = 3	(<i>p</i> = 0.019) <i>N</i> = 8	(<i>p</i> < 0.0001) <i>N</i> = 8	(<i>p</i> = 0.003) <i>N</i> = 6	(<i>p</i> = 0.42) <i>N</i> = 4
Transcriptional regulation							
<i>hif1a</i>	Transcription factor, regulation of OXPHOS to glycolysis shift	1.01 ± 0.04	0.86 ± 0.27	0.96 ± 0.04	1.03 ± 0.09	1.18 ± 0.06	0.96 ± 0.1
hypoxia inducible factor-1		(<i>p</i> = 0.437) 4	(<i>p</i> = 0.179) 5	(<i>p</i> = 0.207) 8	(<i>p</i> = 0.675) 8	(<i>p</i> = 0.001) 6	(<i>p</i> = 0.357) 4
<i>nrf2a</i>	Transcriptional activator of antioxidant and detoxification genes	1.68 ± 0.13	0.55 ± 0.14	1.17 ± 0.09	2.07 ± 0.17	0.84 ± 0.11	1.40 ± 0.4
NF-E2 p45-related factor 2a		(<i>p</i> = 0.026) <i>N</i> = 6	(<i>p</i> = 0.001) <i>N</i> = 6	(<i>p</i> = 0.166) <i>N</i> = 9	(<i>p</i> < 0.0001) <i>N</i> = 9	(<i>p</i> = 0.235) <i>N</i> = 8	(<i>p</i> = 0.792) <i>N</i> = 4
<i>pgc1a</i>	Transcriptional co-activator of mitochondrial biogenesis genes	1.64 ± 0.17	0.71 ± 0.19	0.8 ± 0.03	1.00 ± 0.07	0.69 ± 0.05	0.85 ± 0.14
peroxisome proliferator-activated receptor γ coactivator 1a		(<i>p</i> = 0.005) <i>N</i> = 6	(<i>p</i> = 0.001) <i>N</i> = 7	(<i>p</i> < 0.0001) <i>N</i> = 9	(<i>p</i> = 0.948) <i>N</i> = 9	(<i>p</i> = 0.003) <i>N</i> = 9	(<i>p</i> = 0.268) <i>N</i> = 3
Mitochondrial health and dynamics							
<i>dnm1</i>	Cytosolic GTPase that controls mitochondrial fission	n/a	n/a	0.52 ± 0.04	0.64 ± 0.18	0.70 ± 0.08	0.89 ± 0.12
Dynamin related protein 1				(<i>p</i> = 0.021) <i>N</i> = 3	(<i>p</i> = 0.116) <i>N</i> = 3	(<i>p</i> = 0.043) <i>N</i> = 3	(<i>p</i> < 0.0001) <i>N</i> = 2
<i>nd5</i>	Mitochondrially-encoded OXPHOS Complex I component	1.20 ± 0.08	0.81 ± 0.21	0.76 ± 0.03	1.11 ± 0.07	0.83 ± 0.04	0.70 ± 0.09
NADH dehydrogenase 5		(<i>p</i> = 0.079) <i>N</i> = 6	(<i>p</i> = 0.001) <i>N</i> = 7	(<i>p</i> < 0.0001) <i>N</i> = 10	(<i>p</i> = 0.003) <i>N</i> = 10	(<i>p</i> = 0.007) <i>N</i> = 9	(<i>p</i> = 0.0004) <i>N</i> = 4
<i>opal</i>	Mitochondrial inner membrane fusion and cristae formation	1.48 ± 0.08	0.64 ± 0.17	0.71 ± 0.03	0.89 ± 0.06	0.86 ± 0.05	0.84 ± 0.13
Optic atrophy 1		(<i>p</i> < 0.00001) <i>N</i> = 6	(<i>p</i> < 0.0001) <i>N</i> = 7	(<i>p</i> < 0.0001) <i>N</i> = 9	(<i>p</i> = 0.015) <i>N</i> = 9	(<i>p</i> = 0.048) <i>N</i> = 9	(<i>p</i> = 0.161) <i>N</i> = 3
<i>peo1</i>	Sole mtDNA helicase	1.34 ± 0.09	0.84 ± 0.21	0.92 ± 0.04	1.14 ± 0.08	0.85 ± 0.04	0.75 ± 0.14
Progressive external ophthalmoplegia 1		(<i>p</i> = 0.007) <i>N</i> = 6	(<i>p</i> = 0.058) <i>N</i> = 7	(<i>p</i> = 0.038) <i>N</i> = 9	(<i>p</i> = 0.012) <i>N</i> = 9	(<i>p</i> = 0.007) <i>N</i> = 9	(<i>p</i> = 0.068) <i>N</i> = 3
Cellular stress							
<i>ogg1</i>	Repairs 8-oxoG DNA lesions	0.88 ± 0.04	0.87 ± 0.28	0.90 ± 0.04	1.76 ± 0.2	1.51 ± 0.09	0.92 ± 0.11
8-oxyguanine DNA glycosylase		(<i>p</i> = 0.00041) <i>N</i> = 4	(<i>p</i> = 0.436) <i>N</i> = 5	(<i>p</i> = 0.017) <i>N</i> = 8	(<i>p</i> < 0.0001) <i>N</i> = 8	(<i>p</i> < 0.0001) <i>N</i> = 6	(<i>p</i> = 0.413) <i>N</i> = 4
<i>sod2</i>	Converts superoxide to H ₂ O ₂ within mitochondria	1.11 ± 0.04	0.70 ± 0.17	0.69 ± 0.05	0.57 ± 0.05	0.5 ± 0.05	1.29 ± 0.22
Mitochondrial superoxide dismutase		(<i>p</i> = 0.0003) <i>N</i> = 6	(<i>p</i> = 0.0051) <i>N</i> = 6	(<i>p</i> = 0.0003) <i>N</i> = 9	(<i>p</i> < 0.0001) <i>N</i> = 9	(<i>p</i> < 0.0001) <i>N</i> = 8	(<i>p</i> = 0.07) <i>N</i> = 4

qPCR expression values, normalized to the geometric mean of *l13a*, *ef1a* and/or *B-actin*. Values are presented as the average fold change ± SEM, with (*p*-value) and *N*, relative to the control levels. Significant values are shaded.