Distinct retinal deficits in a zebrafish Pyruvate Dehydrogenase deficient mutant

Abbreviated title: pyruvate dehydrogenase deficient retina

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Abstract

Mutations in ubiquitously expressed metabolic genes often lead to central nervous system specific effects, presumably due to the high metabolic demands of neurons. However mutations in omnipresent metabolic pathways can conceivably also result in cell type specific effects due to cell specific requirements for intermediate products. One such example is the zebrafish *noir* mutant, which we found to be mutated in the *pdhb* gene, coding for the E1 beta subunit of the Pyruvate Dehydrogenase complex. This vision mutant is described as blind and was isolated due to its vision defect related darker appearance.

A detailed morphological, behavioral, and physiological analysis of the phenotype revealed an unexpected specific effect on the retina. Surprisingly, the cholinergic amacrine cells of the inner retina are affected earlier than the photoreceptors. This might be due to the inability of these cells to maintain production of their neurotransmitter acetylcholine. This is reflected in an earlier loss of motion vision, followed only later by a general loss of light perception.

Since both characteristics of the phenotype are due to a loss of acetyl-CoA production by pyruvate dehydrogenase, we used a ketogenic diet to bypass this metabolic block and could indeed partially rescue vision and prolong survival of the larvae. The *noir* mutant provides a case for a systemic disease with ocular manifestation with a surprising specific effect on the retina given the ubiquitous requirement for the mutated gene.
Introduction

Mutations in genes encoding mitochondrial proteins often preferentially affect tissue with high energy demand (Wallace, 1999), in particular the central nervous system with its neurons being among the most energy demanding cells of the animal body. This is notably true for the retina with its mitochondrion rich photoreceptors being among the most metabolically active cells of the vertebrate body (Ames et al., 1992; Laughlin, 2001; Okawa et al., 2008). The study of such metabolic diseases is often hampered by early embryonic lethality in mammals. A case in point is targeted disruption of subunits of the murine pyruvate dehydrogenase complex that links glycolysis with the Krebs (tricarboxylic acid) cycle. These mice show early embryonic lethality and their use to study effects on the nervous system is very limited (Johnson et al., 1997; Johnson et al., 2001). This constraint can be overcome in the zebrafish model. Since early embryogenesis is supported by maternally supplied mRNA and proteins the mutants are not embryonically lethal. Hence the effect of zygotic mutations affecting basic metabolism can be studied on later developing structures including the nervous system.

As part of a large scale screen to isolate chemically induced mutations affecting embryogenesis, two alleles of the mutant noir have been isolated due to their expanded melanophores giving an overall darker appearance (Kelsh et al., 1996). This mutant was shown to be blind in subsequent behavioral and physiological experiments (Neuhauss et al., 1999). In order to decipher the underlying molecular defect in this mutant we performed positional cloning. This revealed a mutation in the gene coding for the E1 beta subunit of the pyruvate dehydrogenase complex (pdhb). A mutant defective in the E2 subunit of the same complex has been linked to the noa (no optokinetic response a) mutant, which was similarly identified in a screen for mutants
affected in visual behavior (Brockerhoff et al., 1995; Brockerhoff et al., 1998; Taylor et al., 2004). Mutations in this complex cause Leigh’s syndrome in humans (Quintana et al., 2009), a progressive neurometabolic disorder characteristic of focal, bilateral lesions in one or more areas of the central nervous system (Leigh, 1951; McKusick et al., 1986).

We investigated the retinal defect in the *noir* mutant in more detail and found defects both in the outer retina and in cholinergic amacrine cells of the inner retina. Intriguingly, different aspects of vision are differentially affected. Motion vision, the basis of the visual behavior used to identify both mutants, is earlier affected than general light perception. Larvae loose their response to motion stimuli before they lose vision in general, as demonstrated by electroretinography and visual behavior experiments. We propose a metabolic model that may account for the selective deficit of cholinergic cells. Providing the mutant larvae with a ketogenic diet to bypass the requirement of acetyl-CoA production by pyruvate dehydrogenase partially rescues the phenotype and enhances survival of mutant larvae.

The *noir* mutant provides an example for a systemic disease with ocular manifestation with a surprisingly specific effect on the retina given the ubiquitous requirement of the mutated gene.
Materials and Methods

Fish maintenance

Zebrafish (*Danio rerio*) were maintained under standard conditions (Brand M. G. M, 2002). The *noir* (*nir<sup>c22</sup> and *nir<sup>p89</sup>* mutation was kept in heterozygous fish which were crossed to obtain homozygous *noir* larvae. Larvae were kept at 28°C in E3 embryo medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl<sub>2</sub>, 0.33 mM MgSO<sub>4</sub>, 10% Methylene Blue). Strains used in this study were: Tuebingen (Tue) (Haffter et al., 1996), WIK (Dahm, 2005), *nir<sup>c22</sup>* and *nir<sup>p89</sup>* (Kelsh et al., 1996), *TG(fli1:EGFP)* (Lawson and Weinstein, 2002).

Mapping

Heterozygous *nir<sup>p89</sup>*/* fish (Tue background) were crossed to wildtype fish of the WIK strain and from the offspring of these crosses homozygous *noir* larvae and siblings were separated and collected. 48 *nir<sup>p89</sup>*/* larvae as well as 48 siblings were used to perform bulked segregant analysis using 192 single sequence length polymorphism (SSLP) markers distributed over the whole genome. Further fine mapping was performed using the total DNA of single homozygous mutant larvae. DNA extraction and PCR were performed as described (Geisler, 2002). Fine mapping was performed using the total DNA of 720 single *nir<sup>p89</sup>*/* larvae.

Western blot

For protein lysates 30 larvae of each condition were collected and stored at -80°C. 100 µl lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM DTT, 1mM EDTA pH 8, 10% Glycerol, 1% Triton-X 100) per 30 larvae was added, the mix was
sonicated, and centrifuged. Supernatant was used for the remaining procedure.

Protein concentration was determined using the DC Protein Assay Kit (Biorad).

Western blot was performed using a monoclonal mouse anti-human PDHB antibody (Abcam, ab55574) in a 1:2000 dilution and a HRP-conjugated goat anti-mouse antibody (Thermo Scientific) for detection. Immunoblots were developed with the ECL system (Super Signal West, Thermo Scientific).

RT-PCR

Total RNA was isolated from 20 eggs of the 1-cell stage using the RNAeasy Kit (Qiagen). RNA was reverse transcribed using the superscript RTII kit (Invitrogen) and oligo dT primers. For PCR amplification a forward primer (TACAGAGGACAGTGAACATGG) and a reverse primer (ATTGTGTCCGGATGGAGG) and an annealing temperature of 60°C was used to amplify a part of the PDHE1beta gene. A part of the β-actin gene was co-amplified using the forward primer AAG CAG GAG TAC GAT GAG TCT G and the reverse primer GGT AAA CGC TTC TGG AAT GAC. Amplification products were visualized on a 1% agarose gel containing Ethidium bromide.

Histology

Larvae were fixed in 4%PFA (paraformaldehyde in phosphate buffer pH 7.4) overnight at 4°C. After dehydration in a graded series of ethanol-water mixtures, larvae were incubated in a 1:1 and then 1:3 ethanol Technovit 7100 (Heraeus Kulzer, Germany) solution for 1h. After infiltration in the technovit solution overnight larvae were embedded in Technovit 7100 polymerization medium and dried at 37°C for 1h.
3 μm thick sections were prepared with a microtome, mounted onto Superfrost slides (Thermo Scientific) and dried at 60°C. Richardson (Romeis) staining (0.5% Borax, 0.5% Azur II, 0.5% Methylene Blue) was performed and the slides were mounted in Entellan (Merck).

A BX61 microscope (Olympus) was used for imaging the slides in the bright field modus.

**Immunohistochemistry**

Paraformaldehyde-fixed larvae (4% PFA in phosphate buffer pH 7.4) were cryoprotected in 30% sucrose in PBS overnight at 4°C and were embedded in cryomatrix (Tissue Tek O.C.T Compound, Sakura Finetek) using liquid N2 to immediately freeze the samples. Sections of 20 μm were prepared at -18°C using a cryostat and were mounted onto superfrost slides (Thermo Scientific). Slides were air dried at RT and stored at -20°C. Before use, slides were thawed at 37°C for 30 min and washed in PBS, pH 7.4 for 10 minutes. Blocking solution (10% normal goat serum, 1% bovine serum albumine, 0.3% Tween-20 in PBS pH 7.4) was applied for at least 2 hours at RT and primary antibodies diluted in blocking solution were incubated overnight at 4°C. The following antibodies were used: mouse anti-GLutamine Synthase (Chemicon International, MAB302) 1:700, rabbit anti-cPKCβI (C-16) (Santa Cruz, sc-209) 1:150, rabbit anti-Thyrosine hydroxylase (Millipore, AB152) 1:1000, goat anti-ChAT (Millipore, AB144P) 1:100, rabbit anti-Serotonin, 5-HT (Sigma, S5545) 1:1000, mouse anti-Parvalbumin (Millipore, MAB1572) 1:1000, mouse anti-PCNA (Clone: PC10, Zymed Laboratories Inc.) 1:150, rabbit anti-caspase 3 (557038, BD Biosciences) 1:200. For ChAT immunolabelling the normal goat serum was omitted from the blocking solution.
The immunoreaction was then detected using fluorescently labeled secondary antibodies (Alexa Fluor 488 goat anti-rabbit, Alexa Fluor 568 goat anti-mouse, Alexa Fluor 488 donkey anti-goat, all from Molecular Probes) diluted 1:1000 in PBS. Slides were coverslipped and imaged in a BX61 microscope (Olympus) using the appropriate filter.

Cell Quantification

z-stacks within a z-distance of 20 μm and a sequential distance of 1 μm were recorded using a BX61 microscope (Olympus) and the Cell-F software (Olympus Soft Imaging Solutions GmbH). Maximum intensity projections were generated and were used for cell counting.

At least 6 animals per condition were used for quantification. With the help of WCIF Image J cell counter plug-in software (NIH, Bethesda, Maryland, USA) stained cells were counted and data were analyzed with the GraphPad Prism 4.00 software (GraphPad Software, San Diego California, USA). For cell quantification of the ganglion cell layer histologically stained sections were used. Cells in a segment of 60° with its origin in the center of the lens and one arm in close proximity along the optic nerve were counted and evaluated. Cell counts of both eyes of a fish were averaged, resulting in one data point per animal.

Electroretinogram

Electroretinograms were recorded as described before (Makhankov et al., 2004). Briefly, larvae were dark adapted for 30 min prior to recording. Than the animal is placed on a sponge soaked with blank E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, 0.33 mM MgSO₄) and the recording electrode with a tip diameter of 20
µm filled with E3 is placed on the cornea while the reference electrode is beneath the animal. Stimuli of 100 ms with interstimulus intervals of 5 s were applied to elicit an ERG response. The light stimulus intensity was 5600 lux or 60 W/m².

For measuring the a- and d-wave, the b-wave was blocked by incubating the animal in 100 µM APB (L-AP-4) (Tocris Bioscience, Ellisville, Missouri, USA) and 200 µM TBOA (Tocris Bioscience, Ellisville, Missouri, USA) (Wong et al., 2004) in E3 medium for 10 to 30 minutes prior to recording. No dark adaptation was carried out when a- and d-wave were measured and a stimulus duration of 1 s was used in order to separate both waves in time.

Optokinetic Response

Zebrafish larvae were immobilized in pre-warmed 3% methylcellulose as described before (Rinner et al., 2005). Vertical black-and-white sine-wave gratings were projected onto the inside of a white paper drum (d= 9 cm) (Mueller and Neuhauss, 2010). The pattern was rotating around the restrained larva placed in the middle of the paper drum at an angular velocity of 7.5 deg/sec, changing the direction with a frequency of 0.3 Hz. Contrast was varied between 0.05 and 1, starting with the highest contrast, followed by a stepwise reduction to the lowest contrast, after which contrast was increased again to 1. In this way, every contrast (except 0.05) was presented two times for 9 seconds each. Before measurements were initiated, the eyes were pre-stimulated for 9 seconds with a contrast of 0.99. The eyes of the larvae were detected by custom-made software based on Lab View 7.1 and NI-IMAQ 3.7 (National Instruments, USA) (Mueller and Neuhauss, 2010). Angular position of the eyes was determined at 5 frames per second and angular velocity of each eye was calculated in real time. Post-experimental data processing and analysis was conducted as described
in Rinner et al. 2005 (Rinner et al., 2005). Graphs were generated using PASW Statistics 17.0 (SPSS Inc., USA).

Visual Motor Response

A similar setup as described in Emran et al. (Emran et al., 2008) was used to measure the visual motor response (VMR). Larvae were placed in individual wells of a 96-well plate (7701-1651, Whatman, USA) containing E3 medium. Mutants and siblings were arranged in a checkerboard manner to avoid positional effects. The wells were illuminated from below with an array of 36 infrared-emitting diodes ($\lambda_{\text{peak}}=880$ nm) shielded by a diffuser. In addition to the IR-diodes, the array contained 36 UV ($\lambda_{\text{peak}}=361$ nm), blue ($\lambda_{\text{peak}}=435$ nm), cyan ($\lambda_{\text{peak}}=500$ nm) and red ($\lambda_{\text{peak}}=630$ nm) LEDs each. The brightness of each color could be controlled by a pulse-width modulator (PWM83, National Control Devices, USA) connected to the serial port of a computer. The larvae were monitored from above using an infrared sensitive CCD-camera (Pike F-032B, Allied Vision Technologies, Germany) equipped with a zoom-lens (C6Z1218-FA, Pentax, Japan) fitted with an IR-pass filter (IF-093-SN1-49, Schneider Kreuznach, Germany).

Each larva was individually detected and tracked by custom-made software based on LabView 7.1 and NI-IMAQ 3.7 (National Instruments, USA). Swimming speed of each larva was calculated in real-time at 20 frames per second (fps), averaged over periods of 1 second and written to disk every second.

The larvae’s movements were tracked during a period of 7 hours while changing the illumination. An initial period of 2 hours darkness (IR-illumination only) was applied to adapt and synchronize the larvae. Subsequently, all UV, blue, cyan and red LEDs were simultaneously switched on and off in intervals of 30 minutes. After the
experiment, an empirically derived threshold was used to recode swimming speed of each larva into a binary activity measure. R version 2.9.2 (www.r-project.org) was used to plot the average activity of 48 larvae.

Phototaxis
An upturned computer screen was used to perform the phototaxis experiment. Two different assays were performed: in the first one the screen was divided into 3 compartments which were colored black-white-black. In between measurements colors were reversed to redistribute the larvae. 20 larvae were measured 4 times one minute each, with one minute redistribution intervals. The numbers of larvae in the white compartment were counted (data not shown).
In the second assay, the screen was divided into a black and a white compartment of equal sizes. A total of 20 larvae was placed in the dark compartment, and after every minute larvae in the white compartment were counted.

Ketogenic Diet
The ketogenic diet mixture was prepared similarly to the one described by Taylor et al. (Taylor et al., 2004). Briefly, 10 mM stock solutions of lauric acid, myristic acid, palmitic acid and phosphatidyl choline (all from Sigma) were prepared in E3 medium and solubilized in a sonification bath. A final diet mixture was prepared in E3 medium containing 100 μM lauric acid, 100 μM myristic acid, 200 μM palmitic acid and 500 μM phosphatidyl choline, in which larvae were raised.
Results

The noir (nir) mutant was originally identified in a large scale mutagenesis screen due to its darker appearance (Kelsh et al., 1996). Expanded melanophores causing the darker appearance in noir and many other visually impaired mutants are an indication of blindness due to lack of background adaptation (Figure 1). Subsequent behavioral and electrophysiological analyses established that these mutants are unable to follow moving stripes with their eyes and having a defective electroretinogram (ERG), confirming blindness (Neuhauss et al., 1999). Two alleles of the noir mutation (tp89a and tc22) were identified with indistinguishable phenotype. Complementation crossings yielded a mendelian inheritance pattern confirming that the noir \(^{tp89a}\) and the noir \(^{tc22}\) mutation are allelic.

Homozygous noir mutant larvae do not display an overt phenotype until 4 dpf (days post fertilization). At 5 dpf expanded melanophores and a reduced baseline motility are observed (Figure 7A). The larvae remain mostly in a lateral resting position on the surface of the water, as the swimbladder is inflated. However, when startled, mutant larvae display a short but normal swimming behavior, indicating that muscles are unlikely to be directly affected in the mutant. At around 7dpf, mutant larvae die.

noir carries a mutation in the pyruvate dehydrogenase subunit E1 beta (pdhb) gene

In order to identify the mutated gene underlying the noir phenotype we performed positional cloning. We tested 192 SSLP markers distributed over the whole genome in a bulked segregant analysis using nir\(^{tp89}\) mutants. By this we were able to locate the mutation between markers z9402 and z821 on chromosome 22 (Geisler, 2002). In order to narrow the critical interval further down, we used a number of expressed sequence tag (EST) sequences, which were mapped to the same genomic interval to
test for single nucleotide polymorphism (SNP) markers. By performing a chromosomal walk we were able to identify a critical interval of about 1.3 centimorgan between markers fk08c11.x1 and zk259j21_T7 by recombination analysis (Figure 2A). A number of candidate genes from this region were identified and cloned from affected and unaffected sibling larvae. Sequencing of these candidate genes revealed a point mutation in the gene coding for subunit E1 beta of the Pyruvate Dehydrogenase complex (PDHB) in the nirtp89 allele, leading to an exchange of a conserved glutamine to leucine at position 157. Additional sequencing of nirjc22 cDNA identified a point mutation resulting in a premature stop codon at position 63, confirming that the mutation underlying the noir phenotype is in the pdhb gene (Figure 2B). The nonsense mutation in nirjc22 is most likely amorphic, as is the missense mutation in nirtp89 given the identical phenotype.

In order to study the effect of the mutation on the protein level, we used a commercially available monoclonal antibody against a small fragment of the human PDHB protein in a Western blot analysis. We detected two bands in lysates of wildtype zebrafish larvae, a higher molecular weight unspecific band (used as an internal loading control) and a lower specific band of the predicted molecular weight. Homozygous noir larvae of the tc22 allele bearing the premature stop codon are completely devoid of Pdhb protein at all analyzed stages (5, 6 and 7dpf), arguing that any residual maternally supplied protein is used up by the time the phenotype becomes apparent (Figure 2C).

Homozygous noir larvae of the tp89 allele exhibiting an amino acid exchange at position 157 of the amino acid sequence do maintain the Pdhb protein although at reduced levels. Since there is no difference in the severity of phenotype between the
two alleles, we conclude that the phenotype in this allele is caused by a non-functional protein rather than by diminished protein levels. The glutamine at position 157 is conserved in all species surveyed (from yeast to human), supporting a crucial functional role of this residue.

As it is remarkable that a mutation in a key metabolic enzyme such as the PDH complex is compatible with life and development of a zebrafish larvae up to 7 days, we hypothesized that embryonic stages are supported by maternally deposited pdhb mRNA or protein. We therefore isolated total mRNA from wildtype zebrafish eggs at the 1-cell stage, before zygotic transcription starts, and reverse transcribed it into cDNA. We succeeded in amplifying pdhb specific fragments, indicating that pdhb mRNA is maternally deposited in the embryo (data not shown). Presumably this maternal mRNA is translated into functional Pdhb protein, supporting embryogenesis.

noir mutant larvae exhibit morphological alterations in the retina

In order to assess retinal morphology of the noir larvae we performed standard histology of the retina. Histological sections of 5 day old noir retina did not show any morphological alterations when compared to wildtype retinae. At 6dpf, histology revealed gaps in the inner nuclear layer (INL) of the noir retina, while there was no significant reduction in cell counts of the ganglion cell layer (GCL) (supplementary Figure 1). Retinal morphology of noir larvae at 7dpf is dramatically altered compared to wildtype, as degeneration is extended to the photoreceptor cell layer (Figure 3). Surprisingly, retinal damage in the noir retina is first observed in cells of the inner nuclear layer and not photoreceptors, as would be expected from a mutation in a key metabolic enzyme given that photoreceptors are the most energy consuming cells of the retina (Steinberg, 1987). A possible explanation could be that the alterations in the
inner retina are due to abnormal blood vessels invading the retina. In the mammalian retina, hypoxia can induce abnormal sprouting of blood vessels into the inner retina (Garino and Gardner, 2005; Kubota and Suda, 2009). Recently, hypoxia-induced retinal angiogenesis has also been shown to occur in the zebrafish (Cao et al., 2008; van Rooijen et al., 2010). We reasoned that reduced energy availability might mimic oxygen depletion. In order to test this hypothesis we analyzed the retinal vasculature, by crossing the noir mutant into the transgenic fli1:EGFP line which exhibits fluorescently labeled blood vessels (Lawson and Weinstein, 2002). We performed a histological analysis using antibodies against the GFP transgene and found no evidence for abnormal sprouting of either choroidal or hyaloid vessels in the mutant (data not shown). These experiments showed that the retinal phenotype of noir is unrelated to changes in the blood supply.

In order to further locate the defect in the mutant inner retina, we surveyed a number of different cell types of the inner retina. Markers for Müller glia cells (Glutamine Synthetase) and bipolar cells (cPKCβ) revealed no alterations both in cell counts and cellular morphology (data not shown).

Since the motion based OKR assay shows deficits before overall changes in retinal morphology is apparent, we assessed whether cholinergic amacrine cells are altered. In the mammalian retina, these cells, also called Starburst amacrine cells, are known to give input to direction-selective ganglion cells (DSGC) and are crucial for motion detection and direction selectivity (Yoshida et al., 2001; Vaney and Taylor, 2002; Masland, 2005; Demb, 2007; Zhou and Lee, 2008).

We therefore quantified cholinergic amacrine cells of 5, 6 and 7 day old noir retinas by immunostaining of choline acetyl transferase (ChAT), a cholinergic amacrine cell specific marker in the retina (Yazulla and Studholme, 2001). In the sibling retina the
cell count increases with development, while in the mutant the count is lower at 5 dpf, which becomes statistically significant at later stages (5dpf sib: 19.53±1.1, mut: 16.74±0.34, p > 0.05, 6dpf sib:22.39±0.68, mut: 18.50±1.0, p < 0.05, 7dpf sib: 26.10±2.5, mut: 21.30±0.9, p < 0.05) (Figure 4). Displaced ChAT-positive amacrine cells in the GCL were not reliably quantifiable in our hands.

In order to determine whether the reduction is specific for cholinergic amacrine cells we additionally investigated other types of amacrine cells in the noir retina that have their cell nuclei at similar locations than cholinergic cells. With the help of different markers for amacrine cells (Avanesov et al., 2005; Yeo et al., 2009) serotonergic (5-HT positive), dopmaninergic (tyrosine hydroxylase (TH) positive) and parvalbumin positive amacrine cells were quantified. No difference between amacrine cells of the noir and their sibling retinas was found at 5dpf. At 6dpf, however, the only significant difference between mutants and siblings was found in cholinergic amacrine cell counts. A general reduction of amacrine cells occurs at 7dpf, pointing to a cell unspecific effect at this stage (Figure 4).

Since we detected neither an increase in apoptosis nor a different proliferation rate up to 6 dpf, we can not distinguish between these two potential mechanisms that lead to cell count differences between mutant and sibling retinas (supplementary Figure 2). However, as only a small fraction of inner retinal cells are cholinergic, a loss of these cells might be not detectable by staining for apoptosis. Nevertheless, we deem it likely that the selective loss of retinal cholinergic amacrine cells accounts for the morphological alterations found in the 6 day old noir retina.

**Electroretinographic analysis of the noir retina**
A morphological change or even cell death is the final stage of a cellular defect that may very well be preceded by physiological changes apparent in a functional assay. Therefore we recorded electroretinograms (ERG), sum field potentials of the retina in response to light, from 5, 6 and 7 day old larvae. At 7dpf, shortly before the whole larva dies, we were unable to evoke a retinal response (Figure 5 A-C).

The ERG composite can be deconstructed into underlying waves that reflect the function of different parts of the retina. The a-wave, the initial negative deflection reflects photoreceptor activation and is notoriously difficult to quantify in larval zebrafish, since it interferes with the larger positive deflection of the b-wave, reflecting ON bipolar cell activation. Hence we pharmacologically blocked ON transmission with a cocktail of the specific metabotropic glutamate receptor group III agonist (APB) and the glutamate transporters blocker (TBOA) (Grant and Dowling, 1996; Wong et al., 2004; Wong et al., 2005a; Wong et al., 2005b) (Figure 5D and E). In this way we recorded strongly diminished a-wave amplitudes, showing that photoreceptors are less sensitive to light at stages where behavioral defects but not morphological defects are apparent. Similar effects are measured for the b-wave and the d-wave, reflecting activation of the OFF response (Figure 5 F-H). The ERG deteriorates over time, probably following the depletion of maternally supplied Pdhb protein.

**Motion vision is selectively affected in noir larvae**

Since we recorded ERG responses at 5 dpf, a stage where mutant larvae show no OKR, we conclude that at 5 dpf the lack of OKR can not solely be due to defects in the outer retina. Hence a motion vision specific defect, as suggested by the reduction of cholinergic amacrine cells may account for the complete loss of motion vision at 5
We therefore investigated visually mediated behaviors that are motion independent and first confirmed earlier reports that starting at 5 dpf no OKR can be evoked (Figure 6A). We also recorded spontaneous eye movements, indicating that indeed a sensory problem underlies the absence of the OKR.

Since zebrafish larvae exhibit positive phototaxis (Burgess et al., 2010) this behavior can be used to test light perception independent of motion cues. We used a choice paradigm where light adapted larvae can choose between an illuminated and dark compartment. Both sibling and *noir* larvae were found to robustly swim towards the bright compartment, indicating that at 5 dpf motion detection is completely abolished but light perception is still functional. *noir* larvae completely lacked positive phototactic behavior at 6 and 7 dpf (Figure 6B). Since this assay involves swimming we can not exclude the possibility that swimming behavior rather than light perception is defective at these stages. Therefore we employed another visual behavior, the visual motor response (VMR) (Emran et al., 2008) which affords less strenuous swimming. In this behavioral paradigm, the larvae’s movements are tracked over time with an infrared sensitive camera, while changing the illumination. The overall locomotor activity of *noir* larvae was reduced, confirming previous observations (Figure 7A). This reduced baseline motility can be rationalized as an energy-saving response of the larvae (Taylor et al., 2004). Another explanation for reduced locomotor activity might rest on reduced neuromuscular signaling. Signaling between nerve and muscle relies on the neurotransmitter acetylcholine, which is only available in limited amounts in the *noir* mutant. Similarly, the zebrafish *bajan* mutant which is mutated in the acetylcholine synthesizing enzyme ChAT, shows compromised motility and fatigue (Wang et al., 2008). We deem it likely that the
reduced locomotor activity in the *noir* mutant, is caused by a combination of reduced energy availability and decreased neuromuscular signaling. Interestingly, locomotor activity recordings show that *noir* larvae still react to light increments and decrements, even at 7dpf where no retinal activity is measurable by ERG recordings (Figure 7B and C).

Taken together these motion independent visual behavior measurements indicate that the early loss of motion vision can not solely be attributed to defects in the outer retina. This conclusion is also supported by the ERG results. Mutants with similar b-wave amplitudes as in 5 dpf *noir* mutants are well capable to show optokinetic behavior (data not shown). In the *noir* mutant at least residual light perception is preserved right until death of the larva.

**A ketogenic diet can rescue the *noir* phenotype**

Inactivation of pyruvate dehydrogenase (PDH) effectively blocks the transition from glycolysis to the tricarboxylic acid (TCA) cycle. However such a metabolic block can be circumvented by providing the larva with fatty acids that can be utilized to produce acetyl-CoA independent of PDH. Such a ketogenic diet has been successfully used to rescue the phenotype of *noa*, a mutant defective in subunit E2 of the PDH complex (Taylor et al., 2004).

Therefore we used a ketogenic diet for *noir* larvae, consisting of lauric acid, myristic acid, palmitic acid and phosphatidyl choline. As expected we could ameliorate the phenotype as documented by ERG and morphological analyses. The ERG b-wave of 5 day old untreated *noir* mutants has an amplitude of about 80μV, while the one of fatty-acid treated 5 day old *noir* mutants is increased to around 150μV. Similarly, at 7dpf untreated *noir* larvae exhibit a flat ERG, whereas fatty-acid
treated 7 day old *noir* larvae have a small ERG b-wave around 50μV (Figure 8B). An improvement of retinal morphology was found after feeding the *noir* larvae with the ketogenic diet observable when comparing the retina of 7dpf untreated *noir* larvae with fatty-acid treated 7 day old *noir* larvae (Figure 8A). Wholes in the INL and ONL are undetectable in these larvae. We were able to prolong survival of the *noir* mutants up to 14 days.
**Discussion**

We have identified a mutation in the E1 beta subunit of the pyruvate dehydrogenase complex, a key enzyme in energy metabolism linking glycolysis to the TCA cycle, in the visual *noir* mutant. Interestingly, the phenotype starts to develop specifically in the retina by a decreased optokinetic response, reduced electrophysiological responses and morphological alterations in the inner nuclear layer. Progressively, the retinal phenotype deteriorates as evidenced by a flat ERG at day 7 and poor retinal morphology additionally affecting the outer nuclear layer (Table 1). This surprising course of phenotype progression with respect to the underlying mutation in the ubiquitously expressed pyruvate dehydrogenase subunit E1 beta led us to study the *noir* phenotype in more detail focusing on retinal defects.

We found that in the *noir* mutant motion based vision is affected more severely than expected from electrophysiological measurements at 5dpf, whereas light perception is preserved at that stage. Quantification of retinal cell types revealed that cholinergic amacrine cells are selectively damaged in the mutant inner retina, whereas other cell types are only later affected. These morphological results are corroborated by behavioral assays, showing that motion vision, as assayed by the optokinetic response, is earlier affected than light perception per se, as assayed by a phototactic assay. Similarly the progressive decrease of light perception is reflected by a progressive decrease in electroretinogram. Although photoreceptors show decreased light responses at 5 dpf, this reduction can not account for the complete loss of optokinetic behavior at that stage.
Based on these observations, we propose a model in which the *noir* retinal phenotype is caused by two distinct mechanisms acting in parallel in the inner and the outer retina. The inner retinal defect, first apparent as a loss of motion vision, is caused by defects in cholinergic amacrine cells, while the outer retinal phenotype involves decreasing photoreceptor cell function.

Studies in the mammalian retina have shown that glucose consumed by photoreceptors via the choroid vasculature and retinal pigment epithelium (RPE) is not necessarily used for glycolysis and subsequent processing in the TCA cycle but is rather used to fuel the pentose phosphate pathway (PPP) (Poitry-Yamate et al., 1995; Tsacopoulos et al., 1998). This pathway is crucial for the photoreceptor cell as it restores the levels of NADPH needed to perform the conversion of all-trans-retinal to all-trans-retinol in the photoreceptor outer segment (for review see (Saari, 2000; Muniz et al., 2007)). Instead of glucose as an energy source, photoreceptors take up lactate released by Müller glia cells in large amounts which is converted to pyruvate by lactate dehydrogenase (LDH) and subsequently fuels the TCA cycle and oxidative phosphorylation leading to ATP production. There are a number of crucial ATP dependent reactions in the photoreceptor, including the maintenance of dark current which requires intense pumping of Na⁺ by the Na⁺/K⁺ ATPase at the level of the inner segment in order to preserve the electrochemical gradient (Steinberg, 1987; Ames et al., 1992; Okawa et al., 2008), the replenishment of GTP by ATP used to produce cGMP (Hsu and Molday, 1994), membrane renewal (Young, 1976) and powering the phototransduction process (Okawa et al., 2008). Since in the *noir* mutant lactate-based ATP production fails photoreceptor cells face a lack of ATP leading to potential deficits in all those reactions.
As a response to a hypometabolic environment, similarly to hypoxia, the photoreceptor cell may undergo metabolic suppression, which is the reduction of the principal ATP-requiring metabolic activity (Steinberg, 1987). Therefore, the question remains, whether it is the limited availability of ATP that causes preventive reduced activity of the photoreceptor cell in order to maintain a more favorable ATP/ADP ratio, or whether the photoreceptor cell activity is impaired as a consequence of ATP lack. Since we find degeneration in the outer retina of noir mutants at later stages, the metabolic block might just have overwhelmed a potential protective mechanism of outer retinal cells, aggravated by the depletion of maternal supplies. Hence, we suggest that the noir mutant phenotype emerging in the outer retina is caused by a lack of energy in photoreceptor cells and progressively leads to vision loss in these mutants (Figure 9).

Pathogenesis of the earlier arising phenotype in the inner retina seems to be more complex and due to its specificity unlikely to be explained by simple energy depletion.

Histology revealed a selective damage to retinal cholinergic amacrine cells (Starburst amacrine cells in the mammalian retina), which are in the mammalian retina implicated in motion detecting vision by giving input to direction selective ganglion cells (DSGC’s) (for review see (Vaney and Taylor, 2002; Masland, 2005; Demb, 2007; Zhou and Lee, 2008)). These cells release GABA and acetylcholine (ACh) simultaneously, thereby exhibiting excitatory and inhibitory properties. While GABAergic contribution to direction selectivity is well accepted, the function of ACh release is still unresolved. A recent report of Lee et al (Lee et al., 2006) showed that
nicotinic ACh transmission is implicated in direction selectivity. Moreover, Ackert et al suggest a role for ACh in generating the OFF responses in ON DSGCs (Ackert et al., 2009).

The existence of cholinergic amacrine cells in the zebrafish retina has been shown by several groups (Yazulla and Studholme, 2001; Arenzana et al., 2005), their functional analogy to Starburst amacrine cells of the mammalian retina, however, has not been established. Studies in the goldfish retina revealed that blockage of the nicotinic Acetylcholine receptors ablates motion depending vision (Mora-Ferrer et al., 2005), suggesting a role for nACh-receptor mediated cholinergic neurotransmission in motion perception. This hypothesis is consistent with our data, showing that the optokinetic response is abolished in noir mutants, suggesting that cholinergic amacrine cell function is impaired and subsequently these cells wither. Behavioral defects are detected earlier than morphological alterations suggesting a sequential functional impairment of the cells followed by structural degradation.

How might acetyl-CoA depletion affect cholinergic production? We propose a model, where diminished levels of acetyl-CoA leads to diminished production of acetylcholine by the enzyme Choline acetyltransferase (ChAT) (Figure 9). This would interfere with cholinergic signaling and possibly disturb motion perception in the Noir mutant and later on lead to the ablation of cholinergic amacrine cells.

The neuromuscular circuitry, another cholinergic system, might be affected as well in the Noir mutant as shown by reduced locomotion and fatigue, a phenotype also found in the zebrafish bajan mutant that has a non-functional ChAT enzyme (Wang et al., 2008). However, we can not experimentally distinguish whether reduced energy or decreased neurotransmitter availability or both factors together lead to compromised locomotion in Noir.
Since both manifestations of the retinal phenotype are ultimately due to the reduced availability of acetyl-CoA, we reasoned that providing the mutant with an alternative source should improve the defects. We could indeed ameliorate the mutant phenotype by providing the larvae with a ketogenic diet of fatty acids. This diet should result in an increase in acetyl-CoA production, bypassing the metabolic block, similar to a previous study of the noa mutant carrying a mutation in the E2 subunit of PDH (Taylor et al., 2004).

The PDH complex is located in the mitochondrial matrix and is considered to be a key metabolic enzyme. All the more does it astonish that zebrafish larvae carrying a mutation in one of the subunits of this enzyme, like noa and noir, survive to rather advanced larval stages. We have detected the existence of maternally deposited pdhb mRNA in the zygote shortly after egg fertilization. With this maternal material the embryo presumably survives the development up to day 7. Not only the diminishing maternal protein levels, but also the concomitant depletion of the yolk sac as an energy source around day 5, enhance the sudden onset of phenotype in both the noa and the noir mutant around this age.

The longer survival of these mutants enables the study of later onset phenotypes, such as the unexpected retinal phenotype. This is in contrast to PDH-deficient mice that are early embryonic lethal (Johnson et al., 1997; Johnson et al., 2001).

In conclusion, the noir retinal phenotype can be described by two parallel pathogenic processes that are both caused by a deficit of acetyl-CoA.
The outer retina is affected by lack of energy, while the inner retinal defect is likely caused by the impaired synthesis of acetylcholine.

Our study extends earlier work on the zebrafish model for PDH deficiency with a more detailed mechanism that leads to vision loss in a second animal model for PDH-deficiency and might help to get a better understanding of the pathogenesis in the retina of human patients suffering from PDH-deficiency. This mutation is a fascinating example of the deletion of a ubiquitous gene function resulting in a cell type specific aberration.
Figure legends

Figure 1

External phenotype of noir mutant larvae

Bright-field images of 5-day-old siblings and homozygous noir mutants. A and C: sibling 5dpf; B and D: noir mutant 5dpf. noir mutants display expanded melanophores (arrow) compared to siblings, indicating defective background adaptation. Scale bar: 100 μm

Figure 2

noir is mutated in the pyruvate dehydrogenase subunit E1 beta (pdhb) gene

A: Schematic representation of the genomic region that contains the noir mutation. Genetic mapping established linkage to SSLP markers z9402 and z821 on chromosome 22. Recombination events (recombination events per number of meioses) are indicated on the left. EST’s that were used as SNP markers are indicated on the right. Three candidate genes (in black font) located within the narrowed area were cloned and analyzed for mutations. B: schematic drawing of the pdhb gene (adapted from Pfam) showing the two transketolase domains. Sequence traces showing the location of the nir tc22 mutation (Y63X) and nir tp89 mutation (Q157L) are depicted. C: Western blot with an anti-human PDHB antibody binding to an unknown epitope located between aa250 and aa360. The zf-Pdhb protein was detected at the predicted molecular weight (arrow), and additionally, an unspecific protein was detected (arrow head) which serves as an internal loading control.

Figure 3
**Standard retinal histology**

A-F: Radial sections of the eye stained with Richardson (Romeis) solution show the appearance of wholes in the mutant inner retina at 6dpf (arrows) and in the outer retina at 7dpf (arrow heads). A: 5dpf sibling; B: 5dpf *noir* mutant; C: 6dpf sibling; D: 6dpf *noir* mutant; E: 7dpf sibling; F: 7dpf *noir* mutant. RPE: Retinal pigment epithelium, ONL: Outer nuclear layer, INL: Inner nuclear layer, GCL: Ganglion cell layer. Scale bar: 50 μm

**Figure 4**

**Quantification of retinal amacrine cells**

In the left panels immunostainings of exemplified 6 day old retinal sections are shown. On the right side, average numbers of serotonergic (5-HT-positive), dopaminergic (TH-positive), cholinergic (ChAT-positive) and parvalbumin positive amacrine cells per 20μm section in 5dpf, 6dpf and 7dpf siblings and mutants are shown (means of n ≥ 6 ± SEM). Statistical analysis was performed with Graph Pad Prism software using Two-way ANOVA. (p < 0.05 for cholinergic 6dpf, cholinergic 7dpf and dopaminergic 7dpf and p < 0.001 for serotonergic and parvalbumin positive 7dpf (INL and GCL) comparing siblings with mutants). AC = amacrine cell, TH = Tyrosine Hydroxylase, ChAT = Choline acetyltransferase, 5-HT = 5-Hydroxytryptamine. Scale bar: 50 μm

**Figure 5**

**Electroretinography**

A-C: Typical ERG traces of siblings and *noir* mutants are shown. A stimulus of 5600 lux for 100 ms was used to elicit saturated responses. A: *noir* mutant and sibling at
5dpf; B: noir mutant and sibling at 6dpf; C: noir mutant and sibling at 7dpf. D-E: ERG traces of siblings and mutants with blocked ON-responses (100 μM APB and 200 μM TBOA). Stimulus of 5600 lux for 1s was used to elicit saturated responses. D: noir and sibling at 5dpf; E: noir and sibling at 6dpf. F-H: Graphs depicting average amplitudes (means of n ≥ 10 ± SEM) of noir mutants and siblings. F: a-wave amplitudes of siblings and noir mutants at 5 and 6 dpf; G: b-wave amplitudes of siblings and noir mutants at 5 and 6 dpf; H: d-wave amplitudes of siblings and noir mutants at 5 and 6 dpf.

Figure 6

Visual behavior

A: Optokinetic Response measurements of noir mutants compared to sibling larvae. The optokinetic responses were triggered by a moving grating of varying contrast. Siblings exhibit increasing eye tracking velocities with increasing contrast, whereas mutants do not show any response, despite spontaneous eye movements. Plotted are means of n = 10 ± SEM, the eye speed is measured in degrees per second and contrast is normalized to maximal contrast. B: Phototactic behavior was assessed by a choice paradigm where light adapted larvae (n=20) can choose between an illuminated and a dark compartment. The number of larvae in the illuminated compartment was evaluated minute-by-minute at different larval stages.

Figure 7

Visual Motor Response (VMR)

The VMR was assessed by tracking the locomotor activity and velocity of 48 larvae over a period of 7 hours. Lights were switched on and off every 30 minutes (off-
periods are shaded in grey). A: Average activity during the whole tracking period is shown. B: Average activity during one minute before and one minute after the OFF-ON switch (light ON-response) is shown. C: Average activity during one minute before and one minute after the ON-OFF switch (light OFF-response) is shown.

**Figure 8**

**Ketogenic diet ameliorates the noir phenotype**

A: Histology of larvae raised in E3 medium containing a mixture of fatty acids is shown. Retinal morphology is improved in diet-treated mutant larvae compared to non-treated mutant larvae. Scale bar: 50 μm. B: Typical traces of ERG recordings of diet-treated larvae and non treated larvae are shown. A light stimulus of 5600 lux and 100 ms was applied to elicit saturated responses.

**Figure 9**

**Metabolism in the noir mutant**

Metabolic model of the retinal defect in noir larvae. Both acetylcholine production and ATP production are reduced when the PDH complex is blocked. β-oxidation of fatty acids remains as a source of Acetyl-CoA, but can not compensate completely for the lack of pyruvate-based Acetyl-CoA synthesis. Therefore, noir mutants are confronted with a decrease of Acetyl-CoA levels along with the depletion of maternal stores leading to cell-type specific effects. Cholinergic cells fail to produce enough neurotransmitter resulting in disturbed signaling, whereas photoreceptor cells can hardly deal with reduced ATP levels as apparent by their reduced function. Ultimately, PDH-deficiency in the noir mutant leads to motion blindness first, followed by decreasing general light perception. PDH = Pyruvate dehydrogenase,
LDH = Lactate dehydrogenase, ChAT = Choline acetyltransferase, OXPHOS = Oxidative Phosphorylation

**Supplementary Figures**

**Figure S1**

**Quantification of retinal ganglion cells**

A: Average numbers of ganglion cell nuclei in a region of interest (ROI) are shown. Statistical analysis using a Two-way ANOVA test revealed no significant difference between the *noir* and sibling retina. B: Exemplified histological section of a 6 dpf *noir* retina. A segment of 60° with its origin in the center of the lens and one branch in close proximity along the optic nerve is indicated as region of interest (ROI). Scale bar: 50 µm. C: An enlargement of the ROI is shown in which the counted cells are marked. Scale bar: 20 µm.

**Figure S2**

**Apoptosis and proliferation in the *noir* retina**

A: Quantification of apoptotic cells in the *noir* retina is shown. Apoptotic cells were stained with a caspase 3 antibody. A Two-way ANOVA test revealed a statistical difference between *noir* and sibling retina only at 7 dpf in the INL and ONL (p < 0.01 for 7d INL and p < 0.05 for 7d ONL). B: Proliferating cells were analyzed by anti-PCNA staining. No significant difference was found between *noir* and sibling retina. GCL = Ganglion cell layer, INL = Inner nuclear layer, ONL = Outer nuclear layer.
References


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Summary of the *noir* phenotype

<table>
<thead>
<tr>
<th></th>
<th>5dpf</th>
<th>6dpf</th>
<th>7dpf</th>
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<tr>
<td><strong>External appearance</strong></td>
<td><em>nir</em> darker than siblings</td>
<td><em>nir</em> darker than siblings</td>
<td><em>nir</em> darker than siblings</td>
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<td>cholinergic amacrine cells</td>
<td>multiple cell types reduced</td>
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<tr>
<td></td>
<td></td>
<td>reduced</td>
<td>(PRCs, ACs, MCs, BPCs)</td>
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<td><strong>ERG</strong></td>
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<td>a-wave more reduced</td>
<td>a-wave lost</td>
</tr>
<tr>
<td></td>
<td>b-wave reduced</td>
<td>b-wave lost</td>
<td>b-wave lost</td>
</tr>
<tr>
<td></td>
<td>d-wave reduced</td>
<td>d-wave more reduced</td>
<td>d-wave lost</td>
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<tr>
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<td>no OKR</td>
<td>no OKR</td>
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<td>no phototaxis</td>
</tr>
<tr>
<td><strong>Locomotion</strong></td>
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<td>locomotion more reduced</td>
<td>almost no locomotion</td>
</tr>
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</table>

PRCs = Photoreceptors, ACs = Amacrine cells, MCs = Müller glia cells, BPCs = Bipolar cells