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Pyridox(am)ine 5′-phosphate oxidase (PNPO) deficiency in zebrafish results in fatal seizures and metabolic aberrations

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ABSTRACT

Pyridox(am)ine 5′-phosphate oxidase (PNPO) catalyzes oxidation of pyridoxine 5′-phosphate (PNP) and pyridoxamine 5′-phosphate (PMP) to pyridoxal 5′-phosphate (PLP), the active form of vitamin B_6. PNPO deficiency results in neonatal/infantile seizures and neurodevelopmental delay. To gain insight into this disorder we generated Pnpo deficient (*pnpo*$^{-/-}$) zebrafish (CRISPR/Cas9 gene editing). Locomotion analysis showed that *pnpo*$^{-/-}$ zebrafish develop seizures resulting in only 38% of *pnpo*$^{-/-}$ zebrafish surviving beyond 20 days post fertilization (dpf). The age of seizure onset varied and survival after the onset was brief. Biochemical profiling at 20 dpf revealed a reduction of PLP and pyridoxal (PL) and accumulation of PMP and pyridoxamine (PM). Amino acids involved in neurotransmission including glutamate, γ-aminobutyric acid (GABA) and glycine were decreased. Concentrations of several, mostly essential, amino acids were increased in *pnpo*$^{-/-}$ zebrafish suggesting impaired activity of PLP-dependent transaminases involved in their degradation. PLP treatment increased survival at 20 dpf and led to complete normalization of PLP, PL, glutamate, GABA and glycine. However, amino acid profiles only partially normalized and accumulation of PMP and PM persisted. Taken together, our data indicate that not only decreased PLP but also accumulation of PMP may play a role in the clinical phenotype of PNPO deficiency.

1. Introduction

Pyridox(am)ine 5′-phosphate oxidase (PNPO; EC 1.4.3.5) catalyzes oxidation of pyridoxine 5′-phosphate (PNP) and pyridoxamine 5′-phosphate (PMP) to pyridoxal 5′-phosphate (PLP), the biologically active form of vitamin B_6 (Fig. 1). PNPO deficiency (OMIM #610090) is a rare autosomal recessive inborn disorder causing early-onset epileptic encephalopathy with rapid deterioration and death if untreated [1,2]. In agreement with the position of PNPO in the human vitamin B_6 metabolic pathway (Fig. 1), the first described cases of PNPO deficiency were responsive to treatment with PLP, but not pyridoxine (PN) [1,2]. The identification of new *PNPO* mutations expanded the clinical phenotype, including responsiveness of seizures to treatment with PN and broadening the age of seizure onset from intrauterine/neonatal to 3 years of age [3–7]. However, the genotype-phenotype relationship is far from clear cut. Other factors, such as premature birth and maternal vitamin B_6 supply are suggested to influence seizure onset age by affecting residual PNPO enzyme activity and/or the availability of PLP [3]. Characterization of recombinant mutant PNPO proteins has indicated that PN-responsiveness could possibly be explained by decreased affinity of PNPO for PNP and/or its cofactor flavin mononucleotide (FMN) [5,8]. Moreover, as demonstrated by *in vitro*

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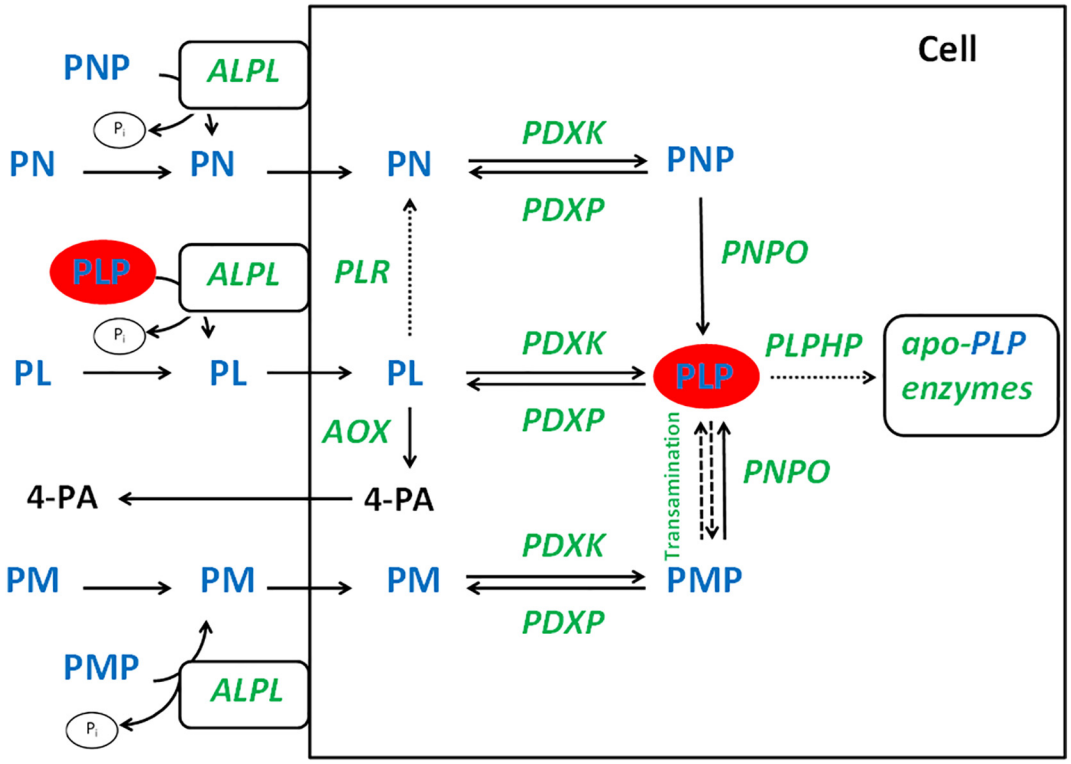

Fig. 1. Schematic representation of vitamin B_6 metabolism.
PN, pyridoxine; PL, pyridoxal; PM, pyridoxamine; PNP, pyridoxine 5′-phosphate; PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; 4-PA, 4-pyridoxic acid; PDXK, pyridoxal kinase; PDXP, pyridoxal phosphatase; PNPO, pyridox(am)ine 5′-phosphate oxidase; PLR, pyridoxal reductase; PLPHP, pyridoxal phosphate homeostasis protein; Pi, inorganic phosphate; AOX, aldehyde oxidase; ALPL, alkaline phosphatase, tissue-nonspecific.

expression studies, most *PNPO* variants yield proteins with residual enzyme activities of varying magnitude [3], which possibly can be boosted by increasing substrate concentrations. A substantial number of affected children show neurodevelopmental delay [3,4,9–11]. While there is a correlation between the genotype and responsiveness to either PLP or PN treatment, the seizure onset age and responsiveness to either PLP or PN are poor predictors of the neurodevelopmental outcome [3,4]. Early initiation of treatment with vitamin B_6 and good seizure control appear to be beneficial [3,9–11]. The reason for the incomplete success of vitamin B_6 treatment is not known.

To date the B_6 database [12] indicates that PLP is a coenzyme in a large number of enzymatic reactions (72 in metazoans and 40 in humans) involved in amino acid and neurotransmitter metabolism, heme biosynthesis, glycogen degradation and other pathways. PLP is essential for normal brain development and function. In humans, genetic defects affecting vitamin B_6 metabolism usually manifest in encephalopathy, seizures and neurodevelopmental delay [13]. Vertebrates, including humans, have no *de novo* synthesis pathways and produce PLP through a salvage pathway (Fig. 1) [14]. Vitamin B_6 is comprised of a group of related compounds (vitamers) including pyridoxal (PL), pyridoxamine (PM) and PN, and their corresponding 5′-monophosphates (PLP, PMP and PNP, respectively). Transport of PL, PM and PN is a passive process aided by intracellular trapping through phosphorylation [15]. Phosphorylated dietary B_6 vitamers (PLP, PMP and PNP) are hydrolyzed by the membrane-bound enzyme alkaline phosphatase (ALPL) prior to entering the cell and crossing the blood-brain barrier (Fig. 1) [16,17]. Intracellularly, PLP is formed from PL by pyridoxal kinase (PDXK), or from PM and PN by the consecutive actions of PDXK and PNPO [14]. Therefore, PNPO is essential in the utilization of meat- and fish-derived (PM(P)) as well as plant-derived (PN(P)) PLP precursors [18]. In humans, *PNPO* expression is highest in the liver, which plays a key role in the interconversion of dietary B_6 vitamers and synthesis of PLP [19], although recent findings suggest an important contribution of the intestine to these processes [20]. Albumin-bound PLP is released from the liver into the circulation and is transported to peripheral tissues and the brain. Pyridoxal phosphatase (PDXP) facilitates the release of B_6 vitamers from cells and regulates intracellular PLP concentrations (Fig. 1) [21,22]. Moreover, pyridoxal phosphate homeostasis protein (PLPHP) has been recently identified as a novel regulator of intracellular PLP homeostasis [23]. Vitamin B_6 is degraded predominantly in the liver and kidney through conversion of PL to 4-pyridoxic acid (4-PA), with subsequent excretion in urine [19]. Aldehyde oxidase (AOX) is thought to be the main enzyme involved, however, recent data from recombinant murine AOX isoform analysis questions this notion at least in mice [24]. Recently, evidence for the conversion of PL to PN by a putative pyridoxal reductase (PLR) has been presented in human cell lines [25].

Our knowledge of the biochemical consequences of PNPO deficiency and the effects of vitamin B_6 treatment is limited to human body fluids (plasma and cerebrospinal fluid (CSF)) [2–4,10,26–31]. To gain more insight in the effects of PNPO deficiency on cellular and tissue levels, we generated *pnpo*$^{-/-}$ zebrafish (*Danio rerio*) and a *PNPO*$^{-/-}$ HEK293 cell line using clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9 gene editing technology. We show that Pnpo deficiency in zebrafish results in reduction of PLP, PL and GABA, accumulation of PMP and PM, accompanied by the development of seizures and decreased survival. Most of these parameters normalized upon PLP treatment, indicating that seizures and lethality in *pnpo*$^{-/-}$ zebrafish are direct consequences of PL(P) deficiency. Lack of normalization of increased PMP in response to PLP treatment suggests that not only decreased PLP but also accumulation of PNPO substrate(s) may play a role in the clinical phenotype of PNPO deficiency and possibly contribute to the long-term complications often observed in PNPO deficient patients.

2. Materials and methods

2.1. Zebrafish maintenance

Animal experiments were approved by and performed according to the guidelines of the animal welfare committee of the Royal Netherlands Academy of Arts and Sciences (KNAW). Zebrafish were raised and maintained under standard conditions [32]. From 6 days post fertilization (dpf) on, zebrafish larvae received a combination of feeds including powdered flake feed (Tetramin, Tetra), dry food feed (NOVO TOM, JBL) and, starting from 8 dpf, also live artemia (*Artemia nauplii*, 24 h after hatching), 3 times a day. From 16 dpf on, zebrafish larvae were fed exclusively live artemia 3 times a day. The analysis of vitamin B_6 in the feeds is shown in Fig. S1. The WT and *pnpo*$^{-/-}$ larvae were treated with 0 μM, 1 μM, 10 μM, 100 μM or 500 μM of pyridoxal 5′-phosphate monohydrate (PLP, Sigma-Aldrich) starting from 16 dpf, for 5 days and 30 min/day between 9.00 and 10.30 am. Treatment included placing zebrafish larvae in plastic tanks containing 500 ml PLP solution at the above indicated concentrations in system water. After 30 min treatment with PLP, zebrafish larvae were gently rinsed and placed in the home tank. Zebrafish larvae were terminated by instantly freezing on dry ice (1 larva per Eppendorf cup) and stored at −80 °C until analysis.

2.2. Larval fin dissections at 3 dpf

Larval fin dissections for genotyping were performed at 3 dpf using a previously published protocol [33] with modifications. Tricaine was added to the embryo medium E3 (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2{\cdot}2H_2O$ and 0.33 mM $MgSO_4$) in advised concentrations [32]. Larvae were transferred to a Petri dish lid using a glass pipette with flame-rounded edges. The tip of the caudal fin was dissected using a disposable microscalpel (nr. 15, Swann-Morton) by applying downward pressure distal from the tail artery. Zebrafish larva was transferred to a 48-well plate (Greiner Bio-one CELLSTAR, 1 larva/well, 1 ml E3/well) using the glass pipet containing E3. The fin tissue was transferred to a PCR plate containing 25 μl Single Embryo Lysis (SEL) buffer (50 mM KCl, 2.5 mM $MgCl_2$, 10 mM Tris pH 8.3, 0.05% NP40 10%, 0.05% Tween-20 and 0.01% gelatin) per well.

2.3. sgRNA and Cas9 mRNA design and synthesis

CRISPR/Cas9 gene-specific regions for *pnpo* were designed by the Sanger Institute (Hinxton, Cambridge, United-Kingdom) using a modified version of CHOPCHOP (http://chopchop.cbu.uib.no). Target sites were selected in exon 2 and exon 3 which match the sequence GG-N21-GG (Supplemental Table S1) [34,35]. The gene-specific oligonucleotides contained the T7 promotor sequence (5′-TAATACGACTCACT ATA-3′), the GGN20 target site without the Protospacer Adjacent Motif (PAM), and the constant complementary region 5′-GTTTTAGAGCTAG AAATAGCAAG-3′. Oligonucleotides were ordered at IDT (Integrated DNA Technologies, Coralville, Iowa, USA) and the zebrafish specific pCS2-nCas9n plasmid was obtained from Addgene (Cambridge, USA). Cas9 mRNA transcription and sgRNA synthesis were performed as described before [36].

2.4. Generation of pnpo knockout zebrafish

Wild type Tupfel longfin (WT TL) one-cell stage zebrafish embryos were microinjected in the yolk with approximately 1 nl sgRNA mixture (sgRNA targeting exon 2 and 3, each 130 ng/μl) and Cas9 mRNA (250 ng/μl) (Fig. 2A). At 4 dpf some of the healthy microinjected larvae were sacrificed to establish CRISPR efficiency. The remainder was raised till adulthood and outcrossed with WT TL. DNA was extracted from healthy embryonal offspring (F1) at 24 dpf to assess for heterozygous variation. Offspring from a mosaic founder that contained an out-of-frame mutation was raised till adulthood and was fin-clipped for genotyping at 9 weeks of age. For the experiments described in this paper, we used F3 WT and homozygous zebrafish, which were obtained from crossing F2 heterozygous fish. Prior to the experiments, we dissected the larval fin from these F3 larvae at 3 dpf as described above. The obtained tissue was used for genotyping and the corresponding larvae were raised, separated per genotype and treatment regime.

2.5. DNA extraction and genotyping

Material for genotyping was obtained from larval fin dissections, whole 1–4 dpf embryos or by fin-clipping adult zebrafish. Larval fins were lysed in 25 μl SEL with 2% freshly added Proteinase K (10 mg/ml). For single embryos or fin-clipped tissue, 50 μl SEL or 100 μl SEL with 2% freshly added Proteinase K was used, respectively. DNA was isolated using the following thermocycler program: 60 min at 60 °C, 15 min at 95 °C, 15 min at 4 °C, infinitely (∞) at 16 °C. Genomic regions flanking the CRISPR target sites were amplified with CRISPR site-specific PCR primers (Supplemental Table S1), using Taq Gold polymerase (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) in combination with a touch down PCR program as previously described [37]. Amplicons were visualized on a 3% agarose gel and mutations were confirmed by Sanger sequencing.

2.6. Behavioral analysis

For behavioral analysis, zebrafish larvae were transferred to a 24-well flat-bottom plate (Greiner Bio-one CELLSTAR) in approximately 2 ml E3 medium and were allowed to acclimatize in the system for 15 min prior to the experiment. Movement of zebrafish larvae was tracked with the Zebrabox system equipped with Zebralab software (Viewpoint Live Sciences, Lyon, France). The following settings were used: background 15 (5 dpf) or 20 (> 5 dpf), inactivity threshold ≤1 mm/s (all ages), and burst activity threshold ≥5 mm/s (5 dpf) or ≥25 mm/s (> 5 dpf). Temperature was maintained at 28 ± 1 °C. Larvae were tracked in the dark without any intervention (1 h at 5 dpf and 1.5–3.5 h in older larvae).

2.7. Generation of PNPO knockout HEK293 cells

Human embryonic kidney 293 (HEK293) cells were transfected with pSpCas9(BB)-2A-GFP (PX458) encoding a sgRNA that targets *PNPO* (NM_018129) (sgRNA3: GTGTTCAGTGTCCTGACATAG). GFP-positive cells were sorted using a FACSAria II flow cytometer (BD) and plated in 10 cm dishes containing DMEM (cat. no. 31966, Gibco, contains ~20 μM pyridoxine HCl) supplemented with 10% heat-inactivated fetal bovine serum (FBS-HI, Gibco) and 1% penicillin-streptomycin (P/S, Gibco). After 1 week, colonies were picked from these plates and clones were expanded in custom-made DMEM (no vitamin B_6, like 31966, Gibco) supplemented with 20 μM pyridoxal HCl (Sigma-Aldrich), 10% FBS-HI and 1% P/S. Clones were tested for expression of the PNPO protein using immunoblotting with a PNPO antibody (sc-82319, Santa Cruz Biotechnology). Genomic DNA from expanded clones was isolated using the QIAamp DNA Micro kit (Qiagen). The targeted genomic regions were amplified by PCR (forward primer: 5′-ACAGTGCCAGGTCC ATAGTAA-3′, and reverse primer: 5′-TTGTGTGTGGATGTATCA AGG-3′), sequenced and analyzed by TIDE (https://tide.nki.nl/). PCR products from candidate knockout lines were cloned into pJET (Thermo Fisher Scientific) and sequenced.

All cells were routinely maintained in custom-made DMEM (no vitamin B_6, like 31966, Gibco) supplemented with 20 μM pyridoxal HCl (Sigma-Aldrich), 10% FBS-HI and 1% P/S in a humidified atmosphere of 5% CO_2 at 37 °C. To determine the effects of PN and PM on vitamin B_6 metabolism, cells were refreshed with custom-made DMEM containing 10% FBS-HI and 1% P/S, in which pyridoxal HCl was replaced with 20 μM pyridoxine (Sigma-Aldrich) or 20 μM pyridoxamine di-HCl

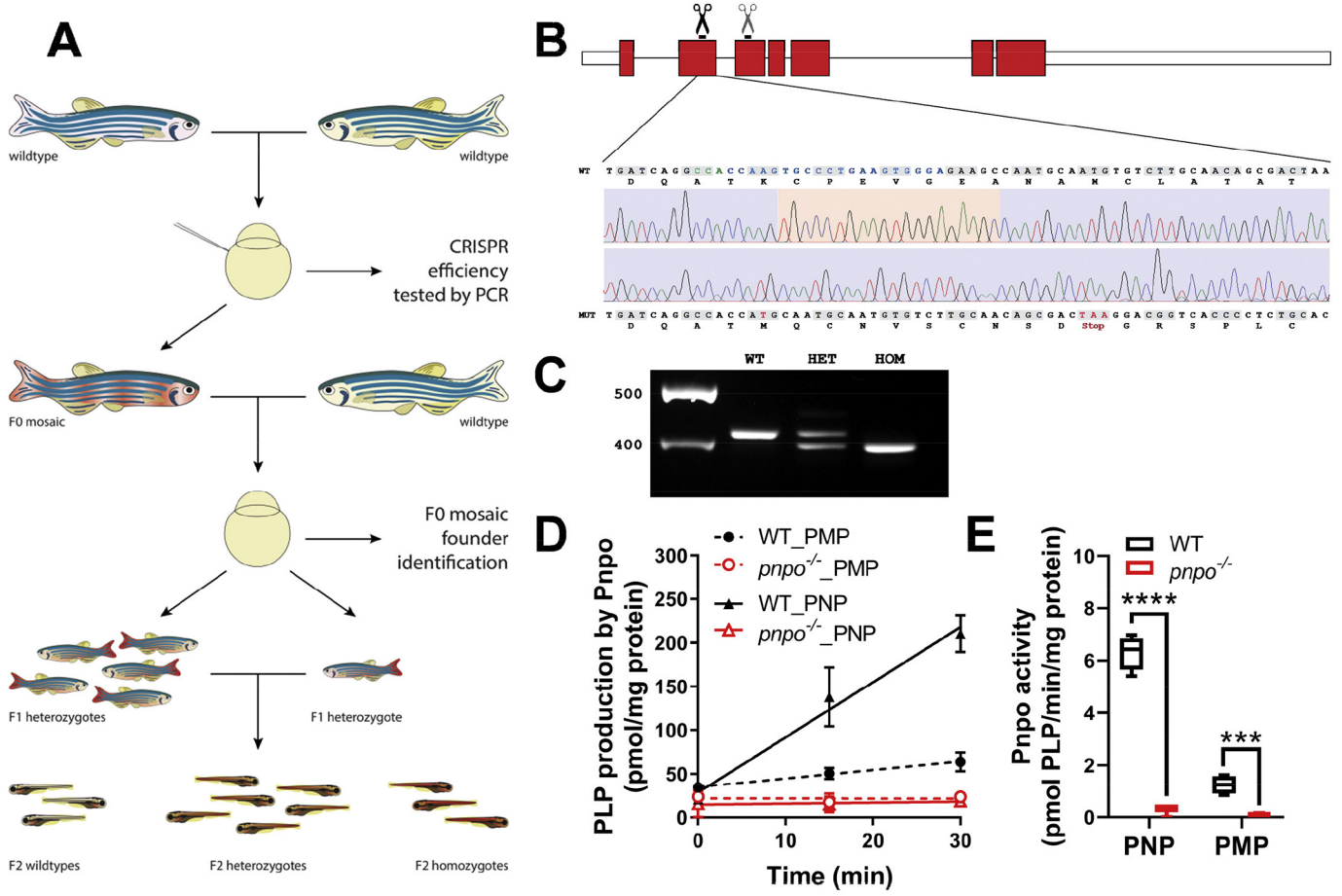

Fig. 2. Generation of the *pnpo*$^{-/-}$ zebrafish line.
A) Schematic overview of the generation of the *pnpo*$^{-/-}$ zebrafish line. Briefly, wild type (WT) TL zebrafish were incrossed and the eggs were injected with sgRNA and Cas9 mRNA in the yolk at the one cell-stage. Some of the injected eggs were sacrificed for genotyping. Adult mosaic zebrafish (F0) were outcrossed with WT TL zebrafish. Some of the embryonal offspring (F1) were used for genotyping, the remainder was raised till adulthood. F1 adults were fin-clipped to identify heterozygous carriers, which were used for breeding. F3 larvae were used for the experiments described in this manuscript.
B) Schematic overview of *pnpo* with Sanger traces of cDNA from wild type embryo pools (*pnpo*$^{+/+}$) and for pools homozygous (*pnpo*$^{-/-}$) for the 20 base pair deletion at the CRISPR site in exon 2 of *pnpo*, which is predicted to result in protein truncation.
C) Representative example of the genotyping PCR visualization by agarose gel electrophoresis for the CRISPR site in exon 2. WT, wild type (*pnpo*$^{+/+}$); HET, heterozygous (*pnpo*$^{+/-}$); HOM, homozygous (*pnpo*$^{-/-}$). Indicated are the amplicon sizes of the DNA ladder.
D) PLP production by Pnpo in zebrafish (20 dpf) homogenates with 400 nM PNP or 400 nM PMP as substrate. Data are means (± SD) from $n = 4$ WT and $n = 3$ *pnpo*$^{-/-}$ zebrafish.
E) Pnpo enzyme activity in WT and *pnpo*$^{-/-}$ zebrafish at 20 dpf. Pnpo activity was calculated from data shown in panel D. ****$p < .0001$ and ***$p = .0002$ (unpaired *t*-test).

(Sigma-Aldrich) and incubated for 48 h. In all experiments, culture medium was refreshed 24 h prior to harvesting the cells.

2.8. B_6 vitamer analysis

Frozen zebrafish larvae were homogenized in ice-cold trichloroacetic acid (TCA; 50 g/l) with zirconium oxide beads (0.5 mm) using a bullet blender tissue homogenizer (Next Advance Inc., Averill Park, NY, USA) at a speed of 8 for 5 min at 4 °C. At 5 dpf, 1 larva was homogenized in 50 µl TCA and extracts from 3 larvae were pooled. At 20 dpf, 1 larva was homogenized in 150 µl TCA. Homogenates were centrifuged at 16200*g* for 5 min at 4 °C. Next, 80 µl of the supernatant (10 times diluted in TCA) was mixed with 80 µl of a solution containing stable isotope-labeled internal standards, vortexed, incubated for 15 min in the dark and centrifuged at 16200*g* for 5 min at 4 °C. The supernatants were used for B_6 vitamer analysis using ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) as previously described [38], except for using 10 times lower concentrations of the calibration samples. B_6 vitamer concentrations (nM) measured at 5 dpf were not normalized to zebrafish weight, since no obvious differences in size between WT, *pnpo*$^{+/-}$ and *pnpo*$^{-/-}$ zebrafish were observed at this age. At 20 dpf, B_6 vitamer concentrations were expressed in pmol per mg of zebrafish wet weight, since considerable variation in size was observed for both WT and *pnpo*$^{-/-}$ zebrafish larvae. B_6 vitamer analysis in HEK293 cell extracts was as in [39]. During all steps, samples were protected from light as much as possible.

2.9. Pnpo enzyme activity

Frozen zebrafish larvae were homogenized in 40 mM Tris-phosphate buffer (pH 7.6) containing 0.1% Triton X-100 (1 larva/150 µl at 20 dpf) using a bullet blender tissue homogenizer (Next Advance Inc.) at a speed of 8 for 5 min at 4 °C. Homogenates were centrifuged at 800*g* for 5 min at 4 °C. The supernatants were diluted 2 times in the same buffer. Pnpo enzyme activity was determined using the method described in [40] with minor modifications. Briefly, 40 µl of diluted supernatants was mixed with 40 µl of reaction mix containing 800 nM PNP (or PMP)

and 3 μM FMN in 40 mM Tris-phosphate buffer (pH 7.6) and incubated for 0, 15 and 30 min at 37 °C with continuous shaking at 500 rpm. At indicated time points, 80 μl of a stop solution containing a mix of stable isotope-labeled internal B_6 vitamer standards in TCA (50 g/l) [38] was added, samples were vortexed and incubated for 45 min on ice in the dark. Samples were centrifuged at 16200*g* for 5 min at 4 °C and supernatants were used to quantify PLP as described previously [38]. Pnpo enzyme activity was calculated as PLP production rate per min per mg of protein. Protein concentrations in zebrafish homogenates were determined using the Pierce BCA protein assay kit (Thermo Fisher Scientific). PNPO enzyme activity in HEK293 cell extracts was determined using the same protocol. To prepare the extracts, cells were cultured in 6-well plates under standard conditions to confluency, washed 2 times with cold DPBS (Gibco) and scraped in 1 ml of 40 mM Tris-phosphate buffer (pH 7.6). Cell suspensions were sonicated for 30 s in the pulse mode (1 s on 1 s off, amplitude 10 μm) on ice, diluted 5 times with 40 mM Tris-phosphate buffer (pH 7.6) and used for the PNPO enzyme assay as described above.

2.10. Amino acid analysis

Frozen zebrafish larvae were homogenized in 100% methanol (1 larva/150 μl at 20 dpf) using a bullet blender tissue homogenizer (Next Advance Inc.) at a speed of 8 for 5 min at 4 °C. Homogenates were centrifuged at 16200*g* for 5 min at 4 °C. Amino acid analysis was performed in undiluted supernatants using a UPLC-MS/MS method [41], except for adapting the range of the calibration samples. At 20 dpf, amino acid concentrations were expressed in μmol per mg of zebrafish wet weight, while at 5 dpf concentrations were not normalized to zebrafish weight, for reasons described above.

2.11. γ-Aminobutyric acid (GABA) analysis

GABA was quantified in zebrafish larvae methanol extracts prepared for amino acid analysis. 100 μl of 1000 times diluted methanol extract was mixed with 20 μl of internal standard (1 μM 4-aminobutyric acid-2,2,3,3,4,4-d6 (GABA-D_6), Sigma-Aldrich) and 500 μl acetonitrile. Samples were vortexed and centrifuged at 16200*g* for 5 min at 4 °C. The supernatants were transferred to a 96-well plate and evaporated to dryness at 40 °C under a stream of nitrogen. Then 100 μl of butylating-reagent (n-butanol:acetylchloride (4:1 (*w*/w))) was added, mixed for 1 min on a plate-shaker and incubated 15 min at 60 °C. Samples were evaporated at 40 °C under a stream of nitrogen and dissolved in 100 μl acetonitrile. The γ-aminobutyric acid (Sigma-Aldrich) standard curve (concentration range 100–500 nM) was prepared the same way. Isocratic separation of GABA was performed on an Acquity UPLC BEH Amide column (130 Å, 1.7 μm, 2.1 × 100 mm) (Waters, Milford, MA, USA) with an Acquity UPLC BEH Amide VanGuard pre-column (130 Å, 1.7 μm, 2.1 × 5 mm). The mobile phase consisted of 10 mM ammonium formate, 0.15% formic acid and 85% acetonitrile and the flow rate was 0.4 ml/min. Column and autosampler temperatures were 30 °C and 10 °C, respectively, and the injection volume was 5 μl. Chromatograms were acquired and processed with Masslynx V4.1 SCN 843 (Waters, Milford, MA, USA). Detection was carried out with a Xevo TQ tandem mass spectrometer (Waters, Milford, MA, USA), which was operated in a positive multiple-reaction-monitoring (MRM) mode. The *m/z* transitions of 160.2 > 87.0 (GABA) and 166.2 > 93.0 (GABA-D_6) were used. The capillary voltage was 0.5 kV and the cone voltage was 18 V. Source and desolvation temperatures were 150 °C and 600 °C, respectively, and cone and desolvation gas flow rates were 2 l/h and 700 l/h. At 20 dpf, GABA concentrations were expressed in pmol per mg of zebrafish wet weight, while at 5 dpf they were not normalized to zebrafish weight.

2.12. Tricarboxylic acid (TCA) cycle intermediates

For analysis, 50 μl of methanol extract (1 zebrafish larva in 150 μl 100% methanol) was mixed with 20 μl of internal standard mix (100 μM) and evaporated under a flow of nitrogen at 40 °C. Next, 25 μl of 0.1% NaOH and 25 μl of O-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine (PFBHA) in Milli-Q water (10 mg/ml (*w/v*)) were added to the dried samples, vortexed and derivatized for 30 min at 4 °C. Calibration samples (concentration range 0.15–80 μM) were prepared by dilution and addition of internal standards. TCA cycle intermediates were separated on a Sunshell RP-Aqua column (150 mm × 3 mm i.d., 2.6 μm; ChromaNik Technologies Inc., Osaka, Japan). The eluents were: solvent A: 0.1% formic acid in H_2O (*v*/v); solvent B: 0.1% formic acid in acetonitrile (v/v). The elution gradient was: 0–2.75 min isocratic 0% B, 2.75–3.5 min linear from 0% to 70% B, 3.5–6.5 min isocratic 70% B, and 6.5–6.7 linear from 70% to 0% B, with 6.7–10 min for initial conditions of 0% B for column equilibration. The flow rate was 0.6 ml/min, column and autosampler temperatures were 40 °C and 10 °C, respectively, and the injection volume was 5 μl. Detection was carried out with a Q-Exactive HF tandem mass spectrometer (Thermo Fisher Scientific). A full scan in a negative ionisation mode (ion source ESI) was performed with a scan range from 70 to 400 *m/z*, resolution 240,000, AGC target of 1e6 and maximum IT of 200 ms. Capillary voltage and temperature were 4 kV and 300 °C, respectively, sheath gas 50, auxiliary gas 20, spare gas 0 and S-lens RF level 65. The m/z transitions were 282.01951 (pyruvate-PFBHA), 285.02957 ($^{13}C_3$-pyruvate-PFBHA), 340.02499 (α-Ketoglutarate-PFBHA), 344.05009 ($^2H_4$-α-Ketoglutarate-PFBHA), 191.01973 (citric acid), 195.04483 ($^2H_4$-citric acid), 117.01933 (succinic acid), 119.02604 ($^{13}C_2$-succinic acid), 115.00368 (fumaric acid), 117.01624 ($^2H_4$- fumaric acid), 133.01425 (malic acid), 136.03308 ($^2H_3$-malic acid), 89.02442 (lactic acid) and 92.03448 ($^{13}C_3$-lactic acid). TCA cycle intermediate concentrations were expressed in nmol per mg of zebrafish wet weight.

2.13. Statistical analysis

Data are presented in box and whiskers plots (maximum, upper quartile, median, lower quartile and minimum) or as means ± SD. Statistical analysis was performed using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). For comparison of two groups, Student's *t*-test was used. For comparison of three or more groups, one-way ANOVA followed by Tuckey's post hoc test was used. To analyze the effects of PLP treatment, two-way ANOVA (independent variables: genotype and PLP treatment) was used. In case of significant interaction between genotype and PLP treatment data were analyzed using one-way ANOVA followed by Tuckey's post hoc test. The level of significance was set at $p < .05$. Information on the specific test used is provided in the figure legends.

3. Results

3.1. Generation of the pnpo knockout zebrafish line

Thirty adult F0 zebrafish were screened for mutations by sequencing their offspring. We identified 1 founder that carried a mutation at the *pnpo* target site in exon 2 (Fig. 2B). Genotyping the adult F1 offspring showed that the founder was 10% germline mosaic, as 6 out of 60 zebrafish were heterozygous (Fig. 2C). Sanger sequencing demonstrated a missense mutation c.119A > T (Zv9) followed by a 20 base pair out-of-frame deletion (c.121_140del), which is predicted to result in protein truncation (p.Met50*). It also showed an intact reference sequence at the CRISPR target site in exon 3. Virtually no Pnpo enzyme activity was detectable in *pnpo*$^{-/-}$ zebrafish at 20 dpf, with either PNP or PMP as substrates (Fig. 2D and E). In WT zebrafish Pnpo enzyme activity was higher with PNP than with PMP at the same substrate concentration, indicating that PNP is the preferred substrate, similarly to bacterial [42]

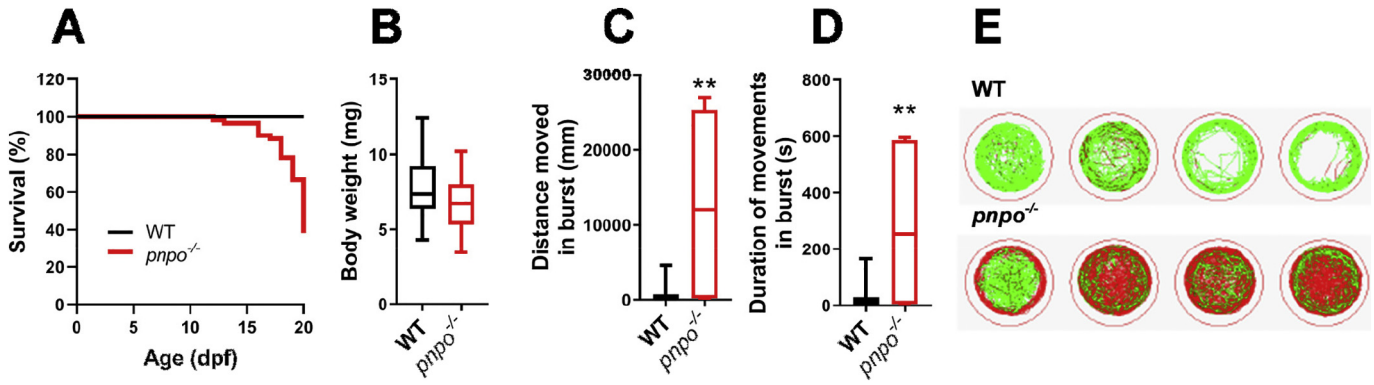

Fig. 3. Phenotypic characterization of *pnpo*$^{-/-}$ zebrafish.
A) Kaplan-Meier curves of WT and *pnpo*$^{-/-}$ zebrafish until 20 dpf. Data are from $n = 44$ WT and $n = 60$ *pnpo*$^{-/-}$ zebrafish.
B) Body weights of WT and *pnpo*$^{-/-}$ zebrafish at 20 dpf. Data are from $n = 19$ WT and $n = 12$ *pnpo*$^{-/-}$ zebrafish.
C) Distance moved in burst and D) duration of movements in burst by WT and *pnpo*$^{-/-}$ zebrafish at 20 dpf. Zebralab software settings were: burst threshold ≥25 mm/s, background 20, duration of tracking 10 min. Data are from n = 12 zebrafish per genotype. $^{**}p < .01$ (*pnpo*$^{-/-}$ compared to WT; unpaired t-test).
E) Representative examples of swimming trajectories of WT and *pnpo*$^{-/-}$ zebrafish at 20 dpf recorded in the tracking mode. Tracked movements are illustrated as burst (red, burst threshold ≥25 mm/s), intermediate (green) and slow (black, freezing threshold ≤1 mm/s). Each circle represents a well on a 24-well plate with a trajectory of a single zebrafish.

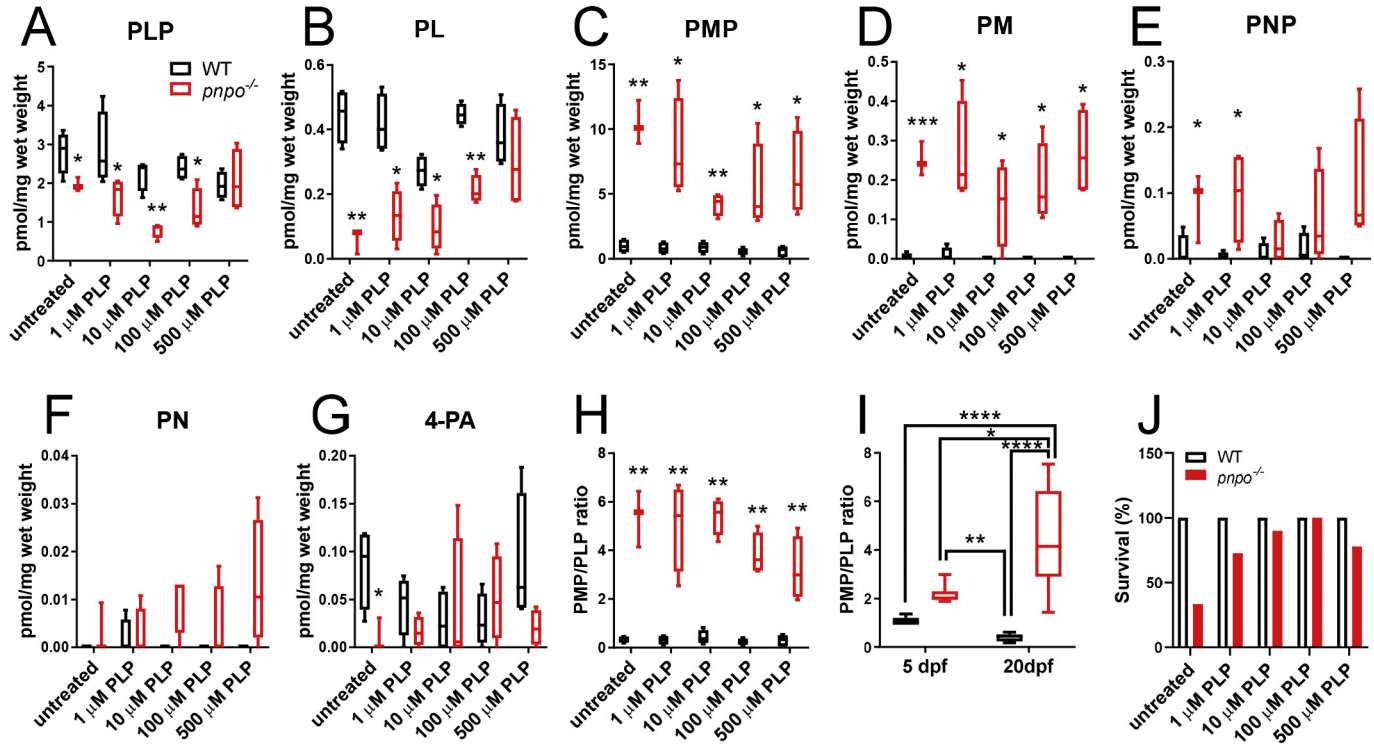

Fig. 4. PLP treatment improves survival and partially normalizes B_6 vitamer profiles in *pnpo*$^{-/-}$ zebrafish.
The effect of different PLP concentrations on A) PLP, B) PL, C) PMP, D) PM, E) PNP, F) PN, G) 4-PA and H) PMP/PLP ratio in WT and *pnpo*$^{-/-}$ zebrafish. Zebrafish were either untreated or treated with 1, 10, 100 or 500 μM PLP from 16 dpf to 20 dpf (5 days), 30 min/day. Data are from n = 4 zebrafish larvae per genotype and treatment group. $^{*}p < .05$, $^{**}p < .01$ and $p^{***} < .001$ compared to WT at the same PLP concentration (unpaired t-test).
I) The effect of age on the PMP/PLP ratio in WT and *pnpo*$^{-/-}$ zebrafish. Data are from n = 6 and $n = 7$–8 zebrafish per genotype at 5 and 20 dpf, respectively. $^{*}p < .05$, $^{**}p < .01$ and $^{****}p < .0001$ compared to WT at 5 dpf or 20 dpf (comparisons indicated on the plot; one-way ANOVA with Tuckey's post hoc test).
J) The effect of different PLP concentrations on the survival of WT and *pnpo*$^{-/-}$ zebrafish. Survival was calculated for the period of treatment and expressed relative to the WT group at the given PLP concentration. Data are from $n = 9$–12 zebrafish per genotype and treatment group.

and human PNPO [40].

Incrossing of zebrafish heterozygous for the *pnpo* mutation (*pnpo*$^{+/-}$) showed a Mendelian inheritance in the offspring up till at least 5 dpf. Survival analysis up to 20 dpf (the latest time point at which biochemical analyses were performed) yielded significantly different survival curves of WT and *pnpo*$^{-/-}$ zebrafish ($p < .0001$, log-rank (Mantel-Cox) test) (Fig. 3A). Based on 3 independent experiments, only 38% of *pnpo*$^{-/-}$ zebrafish survived to 20 dpf compared to 100% survival in WT siblings ($n = 44$ and $n = 60$ for WT and *pnpo*$^{-/-}$, respectively).

3.2. Phenotypic characterization of *pnpo*$^{-/-}$ zebrafish

No difference in mean body weight of WT and *pnpo*$^{-/-}$ zebrafish

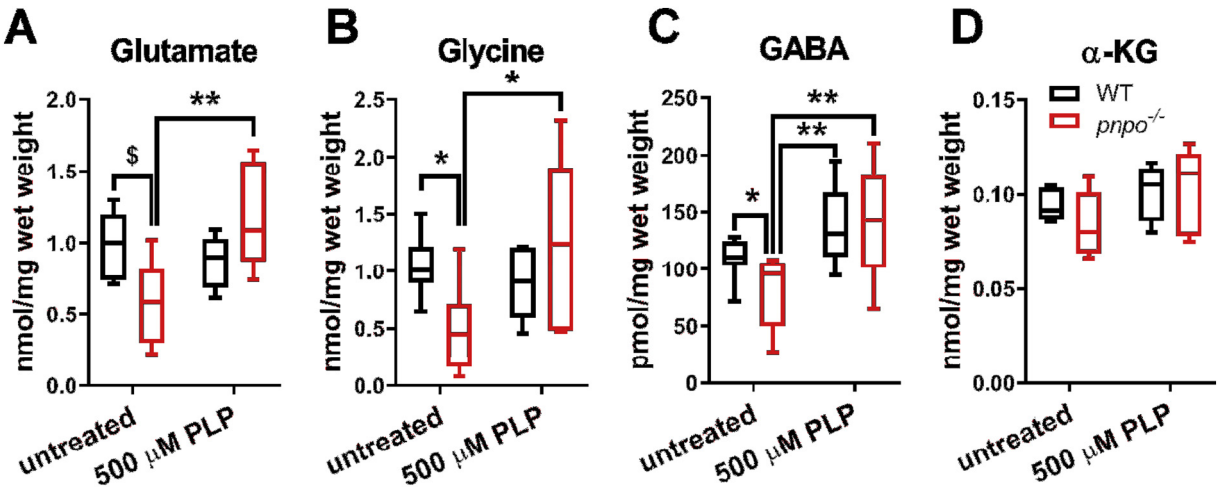

Fig. 5. PLP treatment normalizes glutamate, glycine and GABA in *pnpo*$^{-/-}$ zebrafish.
A) Glutamate, B) glycine, C) GABA and D) α-ketoglutarate in WT and *pnpo*$^{-/-}$ zebrafish at 20 dpf. WT and *pnpo*$^{-/-}$ zebrafish were either untreated or treated with 500 μM PLP from 16 dpf to 20 dpf (5 days), 30 min/day. Data are from n = 4 zebrafish larvae per genotype and treatment group (A) and n = 7 zebrafish larvae per genotype and treatment group (B–E). **p < .01, *p < .05 and $^{\$}p$ = .072 (glutamate) (comparisons indicated on the plot; one-way ANOVA with Tuckey's post hoc test).

was observed at 20 dpf (Fig. 3B). Locomotion analysis showed no spontaneous seizures in *pnpo*$^{-/-}$ zebrafish at 5 dpf (Supplementary Fig. S3H). The first seizing zebrafish were spotted by eye at 16 dpf. However, follow-up experiments showed that the age of seizure onset varies among *pnpo*$^{-/-}$ siblings. Seizures in *pnpo*$^{-/-}$ zebrafish were characterized by an increase in the distance moved in burst (movement speed ≥25 mm/s) and the duration of movements in burst (Fig. 3C–E), both of which are typical locomotion changes described in seizing zebrafish [39,43,44]. The observation of seizing *pnpo*$^{-/-}$ zebrafish indicated that they die within hours after the onset of seizures.

3.3. Pleiotropic alterations in vitamin B_6 and amino acid metabolism in *pnpo*$^{-/-}$ zebrafish

B_6 vitamer analysis at 5 dpf showed that PLP concentration in *pnpo*$^{-/-}$ zebrafish was not significantly different from WT siblings, albeit it tended to be lower (Supplemental Fig. S2A). PL concentration was significantly lower (1.8-fold, $p < .05$), while PM and PMP concentrations were higher compared to WT siblings (Supplemental Fig. S2B-D). The PMP/PLP ratio was 2-fold higher in *pnpo*$^{-/-}$ zebrafish (Supplemental Fig. S2E). Concentrations of 4-pyridoxic acid (4-PA, vitamin B_6 degradation product) and GABA (the main inhibitory neurotransmitter involved in seizures and synthesized by PLP-dependent glutamate decarboxylase), were similar in WT and *pnpo*$^{-/-}$ zebrafish at 5 dpf (Supplemental Fig. S2F and G, respectively). No significant alterations in amino acid profiles were observed at this age (Supplemental Fig. S3).

Analyses in the surviving zebrafish at 20 dpf showed reduced PLP (1.4-fold, $p = .05$) and PL (7-fold, $p < .001$) concentrations in *pnpo*$^{-/-}$ compared to WT zebrafish (Fig. 4A and B, respectively; untreated groups). Furthermore, accumulation of PMP (10.8-fold, $p < .001$) (Fig. 4C, untreated groups), PM and PNP (Supplemental Fig. 4D and E, untreated groups), and lower 4-PA (Fig. 4G, untreated groups) were observed in *pnpo*$^{-/-}$ zebrafish, with no effect on PN (Fig. 4F, untreated groups). The PMP/PLP ratio was 16.2-fold higher ($p < .001$) in *pnpo*$^{-/-}$ compared to WT zebrafish (Fig. 4H, untreated groups). The effect on the PMP/PLP ratio was exacerbated by age (Fig. 4I).

Amino acid analysis at 20 dpf revealed multiple alterations in *pnpo*$^{-/-}$ zebrafish (Supplemental Table S2). Concentrations of the essential amino acids lysine, arginine, tryptophan, methionine, phenylalanine and threonine and concentration of non-essential amino acid tyrosine were elevated in *pnpo*$^{-/-}$ compared to WT zebrafish, indicating decreased activity of PLP-dependent enzymes involved in their degradation. The concentrations of non-essential amino acids glutamine, glutamate, glycine and taurine were negatively affected by Pnpo deficiency. The decrease in taurine and glycine can be attributed to decreased biosynthesis, which involves PLP-dependent enzymes. Due to the involvement of glutamate (and its precursor glutamine) in multiple metabolic pathways, it is difficult to pinpoint single cause for its decrease in *pnpo*$^{-/-}$ zebrafish.

3.4. Normalization of PLP content and improved survival of *pnpo*$^{-/-}$ zebrafish upon PLP treatment

Lower availability of PLP is thought to underlie the phenotype of PNPO deficiency. Therefore, we treated WT and *pnpo*$^{-/-}$ zebrafish with 1, 10, 100 or 500 μM PLP, starting from 16 dpf and for 5 days, 30 min/day. Improvement of survival during the 5 days of PLP treatment was already observed at 1 μM PLP, while treatment with 100 μM PLP resulted in 100% survival of *pnpo*$^{-/-}$ zebrafish (Fig. 4J). However, only treatment with 500 μM PLP resulted in complete normalization of PLP (Fig. 4A), PL (Fig. 4B) and 4-PA concentrations (Fig. 4G). From all B_6 vitamers, only PL in *pnpo*$^{-/-}$ zebrafish correlated strongly with the PLP dose (Pearson $r = 0.892$, $p < .05$). PLP treatment did not prevent accumulation of PMP (Fig. 4C) and PM (Fig. 4D), and did not normalize PMP/PLP ratios (Fig. 4H).

Since treatment with 500 μM PLP resulted in normalization of PLP content in *pnpo*$^{-/-}$ zebrafish (Fig. 4A), we assessed the effect of this dose on amino acid metabolism. Glutamate (Fig. 5A, Supplemental Table S2), glycine (Fig. 5B, Supplemental Table S2) and GABA (Fig. 5C) normalized in *pnpo*$^{-/-}$ zebrafish upon PLP treatment. Irrespective of genotype, PLP treatment resulted in significant elevation of aspartate and branched chain amino acids (Supplemental Table S2). PLP treatment did not result in normalization of lysine, arginine, tryptophan, methionine, phenylalanine, threonine and tyrosine in *pnpo*$^{-/-}$ zebrafish (Supplemental Table S2). Degradation pathways of most of the elevated amino acids involve transaminases that require α-ketoglutarate (α-KG) as the second substrate and decreased availability of α-KG could affect the activity of these enzymes. However, the analysis of TCA cycle intermediates showed that α-KG concentrations were similar in *pnpo*$^{-/-}$ and WT zebrafish (Fig. 5D, Supplemental Table S3). In addition, most amino acids are converted into TCA cycle intermediates and therefore a defect in amino acid metabolism could affect concentrations of TCA cycle intermediates. Further analysis of TCA cycle intermediates

showed that lactate was decreased ($p < .05$) and pyruvate ($p = .064$) tended to be lower in untreated *pnpo*$^{-/-}$ zebrafish, suggesting decreased glycolysis. Impaired glucose utilization has been also reported in knock-down of *sgll/PNPO* in *Drosophila melanogaster* [45]. On the other hand, plasma lactate is often, although not always, elevated in PNPO deficient patients [2–4], possibly suggesting species-dependent responses to PNPO deficiency. Irrespective of genotype, PLP treatment resulted in a significant increase in lactate and succinate concentrations, pointing to interference of PLP with mitochondrial metabolism (Supplemental Table S3).

3.5. Additional insights from a PNPO deficient HEK293 cell line

Due to the small size of the zebrafish larvae, biochemical analyses are performed in a complete organism, averaging out tissue-specific responses to Pnpo deficiency. Moreover, in the present study, zebrafish feeds contained a mix of B_6 vitamers (Supplemental Fig. S1), making data interpretation more complex. To study the consequences of PNPO deficiency in a homogeneous cell population that can be supplied with a single B_6 vitamer, we generated a PNPO deficient human embryonic kidney (HEK293) cell line (Supplemental Fig. S4A and B). PNPO was absent at the protein level and mRNA levels were decreased (Fig. 6A), most likely as a result of nonsense mediated decay. In line with this, in vitro PLP production by PNPO from either PNP or PMP as substrate was not detectable (Fig. 6A and Supplemental Fig. S4C).

We first analyzed B_6 vitamer profiles in WT and *PNPO*$^{-/-}$ HEK293 cells grown with 20 μM PL, PN or PM in the culture medium for 48 h (Fig. 6). It should be noted that prior to this experiment all cells were standardly subcultured with 20 μM PL. As expected, *PNPO*$^{-/-}$ cells grown in 20 μM PL had B_6 vitamer profiles comparable to WT cells, with the exception of elevated intracellular PL concentrations (Fig. 6B). Switching cells from culture medium containing 20 μM PL to culture medium containing 20 μM PN for 48 h caused almost complete depletion of PL (Fig. 6B) and a strong reduction of PLP (Fig. 6C), accompanied by accumulation of PNP and PN (Fig. 6D and E, respectively) in *PNPO*$^{-/-}$ cells. In turn, switching cells from 20 μM PL to 20 μM PM in culture medium resulted in decreased PL (Fig. 6B) and accumulation of PMP and PM (Fig. 6F and G, respectively), similar phenomena as observed in *pnpo*$^{-/-}$ zebrafish larvae, in which PMP and PM constituted a large proportion of nutritional vitamin B_6 (Fig. S1). Unexpectedly, PLP was similar in *PNPO*$^{-/-}$ and WT cells cultured in the presence of PM, which was in sharp contrast to the situation when cells were cultured in the presence of PN (Fig. 6C). Although the precise mechanism of the differential response to PM and PN in *PNPO*$^{-/-}$ cells is not clear, our data indicate that formation of PL(P) from PM(P) (but not PN(P)) is possible via other route than PNPO. We measured total activity of the PLP-dependent enzyme glutamic-oxaloacetic transaminase (GOT) as a readout of altered PLP status in cells grown in 20 μM PL, PM or PN for 48 h (Fig. 6H). In agreement with the effects of different extracellular PLP precursors on intracellular PLP, culturing in the presence of PN only resulted in significantly lower total GOT activity in *PNPO*$^{-/-}$ compared to WT cells.

To address the question whether accumulation of the PNPO substrates PMP and PNP can be influenced by modulating vitamin B_6 supply, we cultured cells with varying concentrations of PM or PN for 48 h and analyzed their B_6 vitamer profiles (Supplemental Fig. S5).

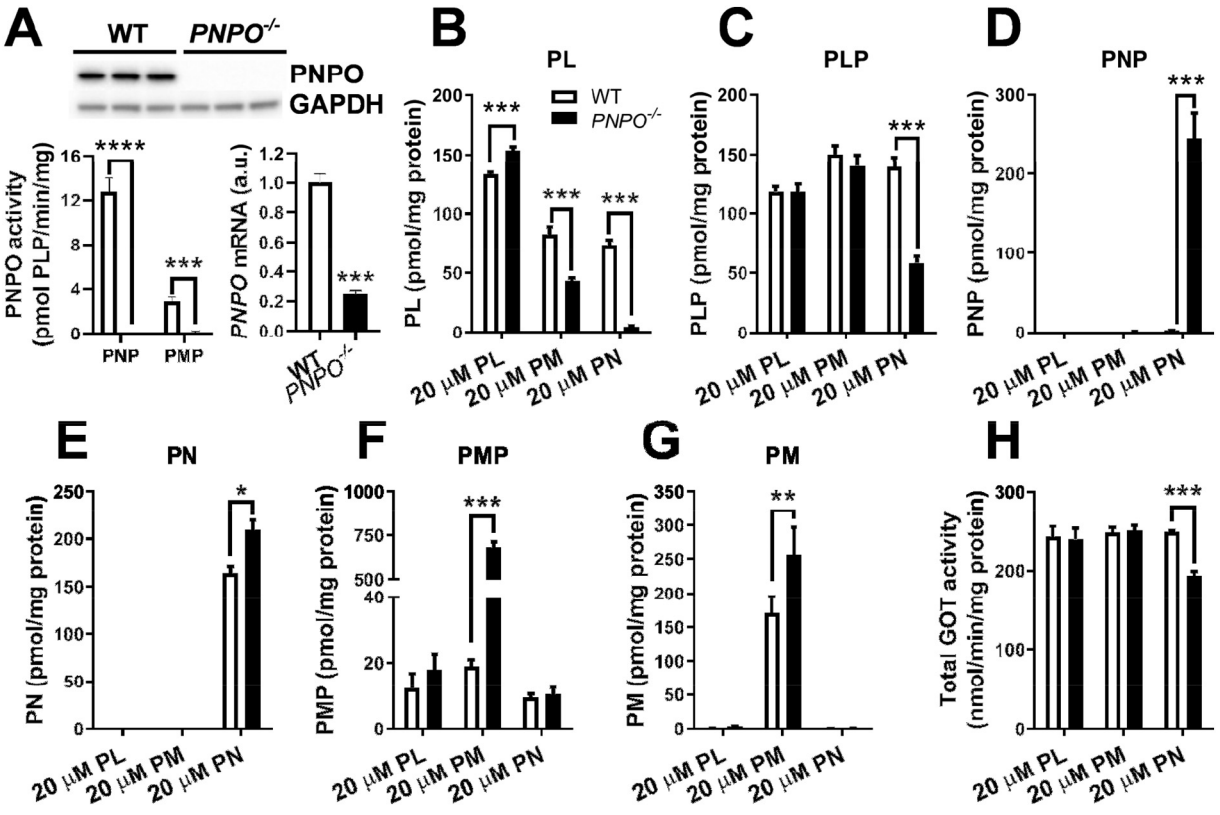

Fig. 6. Effects of PNPO deficiency on vitamin B_6 metabolism in HEK293 cells.
A) PNPO mRNA and protein levels, and PNPO enzyme activity in WT and *PNPO*$^{-/-}$ HEK293 cells. Relative expression of *PNPO* mRNA was quantified relative to the *RPLP0* mRNA using the ΔΔCt method. Protein levels were measured using western blot and quantified relative to GAPDH protein levels. PNPO enzyme activity was measured in cell lysates with 400 nM PNP or 400 nM PMP as substrates. Data are means ± SD from n = 6 (mRNA) and n = 3 (western blot and PNPO activity) biological replicates per genotype. ***$p < .001$ and ****$p < .0001$ (*PNPO*$^{-/-}$ compared to WT; unpaired t-test).
B) PL, C) PLP, D) PNP, E) PN, F) PMP, G) PM and H) enzyme activity of the PLP-dependent enzyme glutamate-oxaloacetate transaminase (GOT) in WT and *PNPO*$^{-/-}$ HEK293 cells grown in 20 μM PL, 20 μM PM or 20 μM PN in the culture medium for 48 h. Data are means ± SD from n = 6 biological replicates per genotype and experimental condition. ***$p < .001$, **$p < .01$, *$p < .05$ (PNPO$^{-/-}$ compared to WT with the same B_6 vitamer in the culture medium; unpaired t-test).

There was a concentration-dependent accumulation of PMP (Fig. S5B) and PNP (Fig. S5E) in *PNPO*$^{-/-}$ cells cultured in PM and PN, respectively. The accumulation of PMP was stronger than that of PNP, which is in agreement with an almost 2-fold lower affinity of human pyridoxal phosphatase (PDXP) for PMP compared to PNP [46]. Intracellular PLP remained quite stable between 5 and 20 μM PM or PN in the culture medium (Fig. S5A and D, respectively), while the capacity to produce PL(P) from PM(P) (Fig. S5A and C) but not PN(P) (Fig. S5D and F) in *PNPO*$^{-/-}$ cells became even more apparent.

4. Discussion

Here we present the first zebrafish model of PNPO deficiency generated using (CRISPR)/Cas9 gene editing technology, which reproduces both biochemical and clinical phenotypes of human disease, including the development of seizures. We show that PLP treatment is effective in normalizing PLP, glutamate, glycine and GABA content and in extending the survival of *pnpo*$^{-/-}$ zebrafish. Our data suggest that while PL(P) is essential in seizure control and survival, accumulation of PNPO substrates (PMP and/or PNP) may play a role in the clinical phenotype of PNPO deficiency and possibly contributes to the incomplete success of PLP treatment.

Decreased availability of intracellular PLP is a common denominator in several genetic defects affecting human vitamin B_6 metabolism, including PNPO deficiency, pyridoxine-dependent epilepsy (caused by mutations in *ALDH7A1* coding for α-aminoadipic semialdehyde dehydrogenase), and epilepsy caused by mutations in pyridoxal phosphate binding protein (*PLPBP*) and tissue-nonspecific alkaline phosphatase (*ALPL*), which all present with encephalopathy, often involving seizures [13]. For this reason, a low level of PLP in CSF is not a specific marker for PNPO deficiency [26,47]. In fact one PNPO-deficient patient with normal PLP in CSF has been reported [27].

PLP is essential in amino acid and neurotransmitter metabolism. Therefore, it is indispensable for normal brain development and function. We have shown previously that in CSF of healthy subjects, PLP and PL are the predominant B_6 vitamers, while PM is present only in low concentrations and PN is not detectable [38,48,49]. Decreased concentrations of PLP [2,10,26,28] and PL [2] in CSF of untreated PNPO deficient patients have been reported, with no information on the other B_6 vitamers. In agreement with these data, we show that PLP and PL were strongly decreased in *pnpo*$^{-/-}$ zebrafish at 20 dpf. The milder effect observed at 5 dpf could be explained by the fact that the yolk containing maternal vitamin B_6 is depleted at 5–6 dpf. Similar to the human situation, in which treatment of PNPO-deficient patients with PLP results in normalization of PLP in CSF [2,10,26], treatment of *pnpo*$^{-/-}$ zebrafish with PLP resulted in normalization of PLP and PL and in increased survival. Similarly, *PNPO*$^{-/-}$ HEK293 cells grown with PL in the culture medium had B_6 vitamer profiles comparable to WT cells, including normal PLP concentrations. It has been shown that PLP treatment can lead to strong elevations of PM in CSF [29] and that plasma PM concentrations are elevated in PNPO-deficient patients irrespective of vitamin B_6 treatment status [50]. We observed accumulation of PM in untreated and PLP-treated *pnpo*$^{-/-}$ zebrafish, and *PNPO*$^{-/-}$ HEK293 cells grown with PM in the culture medium, indicating that PM elevation is a feature of PNPO deficiency.

In this study, PMP was as abundant as PLP at 5dpf and was the second most abundant B_6 vitamer (after PLP) at 20 dpf in WT zebrafish. PMP is almost not detectable in plasma of healthy humans [30,48] and rodents [16,51] and it is not detectable at all in CSF of healthy humans [38,48,49]. This is in agreement with the notion that PMP in body fluids should be rapidly dephosphorylated by ALPL in order to be taken up by tissue cells. Concentrations of PMP in healthy WT rodent tissues are as high or even higher than concentrations of PLP, depending on the type of tissue [16,51], suggesting that PMP measured in our zebrafish extracts is tissue-derived. Presumably, the bulk of intracellular PMP is generated during the catalytic cycle of transaminases, which comprise a large group of PLP-dependent enzymes involved in amino acid metabolism. The general transamination reaction proceeds in two steps: i) formation of an α-keto acid product and PMP (from PLP), followed by ii) formation of an amino acid product and regeneration of PLP (from PMP) [52]. The contribution of transaminases to tissue PMP is supported by the observation that PMP/PLP ratios are distinct in different rodent tissues and are hardly influenced by dietary vitamin B_6 [51]. We observed comparable PMP concentrations in WT HEK293 cells independent of whether they were supplied with PM, PN or PL in the culture medium. This further indicates that under WT conditions, PMP originates from the continuous activity of transaminases. Pnpo deficiency in zebrafish on the other hand resulted in strong accumulation of its substrates PMP and PNP, although quantitatively, PNP concentrations remained negligible. Much stronger accumulation of PMP could be explained by the fact that zebrafish feeds (from 16 dpf on only artemia) contained much more PM(P) than PN(P). Our data suggest that dietary B_6 vitamers may affect intracellular B_6 vitamer profiles in PNPO deficiency. B_6 vitamer profiles of *PNPO*$^{-/-}$ HEK293 cells cultured with PM, PN and PL support this notion. Unfortunately, the influence of dietary vitamin B_6 on the clinical phenotype of PNPO-deficient patients has never been investigated. Information on the effects of PNPO deficiency on vitamin B_6 metabolism in human tissues is available for the liver of a single PLP-treated PNPO deficient patient with cirrhosis, showing elevated content of PL and PA with no information on phosphorylated B_6 vitamers [53]. Therefore, our *pnpo*$^{-/-}$ zebrafish model provides the first insights in the effects of PNPO deficiency in animal tissues, showing that substrates of PNPO strongly accumulate and that this abnormality is not resolved by PLP treatment. The importance of accumulating PNPO substrates is supported by observations in plants, which utilize both de novo and salvage pathways of PLP synthesis. Mutations in *pdx3*, the orthologue of human *PNPO* in plant *Arabidopsis thaliana*, result in accumulation of PMP, induction of stress-response pathways, abnormal amino acid metabolism and growth, with minimally affected PLP [54]. We show that in *pnpo*$^{-/-}$ zebrafish larvae, PLP treatment led to normalization of PLP but did not prevent PMP accumulation. This was accompanied by normalization of only glutamate and glycine, while several, mostly essential, amino acids remained elevated, suggesting a defect in their degradation. Catabolism of amino acids involve a transaminase (lysine and tryptophan require α-aminoadipate aminotransferase/kynurenine aminotransferase II, phenylalanine and tyrosine require tyrosine transaminase, arginine requires ornithine δ-aminotransferase, for example). A negative effect of high PMP concentrations on the activity of transaminases was already postulated in *pdx3* mutants of *A. thaliana* [54]. However, a direct inhibitory effect of PMP on the activity of transaminases is not likely, because during the catalytic cycle of the enzyme, PLP-PMP-PLP transitions occur in the active site of the enzyme and transaminases do not bind PMP from the environment [52]. Our data from *PNPO*$^{-/-}$ HEK293 cells cultured with PM showed that while cells accumulated massive amounts of PMP, the total GOT activity (a representative transaminase) remained comparable to WT cells. The strong decrease observed in GOT activity in *PNPO*$^{-/-}$ HEK293 cells cultured with PN suggests that the activity of transaminases is largely dependent on cellular PLP, which was strongly decreased in these cells.

Alternatively, failure of PLP treatment to normalize concentrations of the abovementioned essential amino acids in *pnpo*$^{-/-}$ zebrafish may be an indication of a more general tissue damage, possibly caused by PMP accumulation. Liver abnormalities, including hepatomegaly [2], abnormal liver function tests [31,53,55] and sometimes even hepatic cirrhosis [53,55], are common in PNPO-deficient patients, although it is not clear whether these abnormalities are an inherent feature of PNPO deficiency or whether they are caused by toxicity of PLP treatment itself. While in our study we cannot discriminate between different tissues due to the small zebrafish size, we could argue that *pnpo*$^{-/-}$ zebrafish display signs of defective liver metabolism based on for example elevated arginine and methionine concentrations, which are

predominantly degraded in the liver.

An intriguing observation was the completely normal PLP level in *PNPO*$^{-/-}$ HEK293 cells cultured in PM, in contrast to cells cultured in PN, where PLP was drastically reduced. This suggests that at least in some (human) cells an enzyme other than PNPO can lead to formation of PL(P) from PM(P). Hypothetically, at high concentrations PMP, which is a primary amine, could be oxidized by another oxidase than PNPO, resulting in formation of PL(P) as part of the PMP detoxification process. Candidate enzymes could be amine oxidases, for example monoamine oxidases with broad substrate specificity [56]. However, this requires further investigation. The fact that despite the abundance of PMP and PM in zebrafish feeds, PLP was decreased in *pnpo*$^{-/-}$ zebrafish, can be explained by relatively lower accumulation of PMP in *pnpo*$^{-/-}$ zebrafish compared to *PNPO*$^{-/-}$ HEK293 cells (10.8-fold and 35.8-fold, respectively). The validity of this notion can be tested by treating *pnpo*$^{-/-}$ zebrafish with high concentrations of PM(P).

Similar to PNPO deficient humans and *Drosophila melanogaster* [57], *pnpo*$^{-/-}$ zebrafish developed severe seizures. The seizure onset was not neonatal, most likely due to the fact that some maternal vitamin B_6 was available to the mutant fish until the yolk was depleted around 5–6 dpf. We noted that the onset of seizures varied considerably among *pnpo*$^{-/-}$ siblings. This may be partially explained by the fact that zebrafish received a mix of feeds with varying vitamin B_6 composition and that some fish likely consumed more than others (based on variation of body weight). Seizures in *pnpo*$^{-/-}$ zebrafish led to death in a time window of a few hours. Treatment with PLP improved the survival rate drastically, presumably by abolishing the seizures through normalization of glutamate (excitatory neurotransmitter, and precursor of GABA), GABA and glycine (both inhibitory neurotransmitters). GABA has been previously implicated in seizures in zebrafish [44] and *D. melanogaster* [57]. Low concentration of glycine has been shown to exert pro-convulsive effect in drug-induced epilepsy in rats with attenuation of convulsions at higher glycine concentrations [58], showing the importance of glycine in regulating brain function.

5. Conclusions

In conclusion, we show that zebrafish can be successfully used to model human disease. Our data from *pnpo*$^{-/-}$ zebrafish and *PNPO*$^{-/-}$ HEK293 cells provide new biochemical insights in PNPO deficiency. This study also shows that further research is needed to completely uncover the genotype-phenotype relationship in PNPO deficiency, including the effects of PMP accumulation. In turn, deeper understanding could provide the basis for better treatment strategies.

Transparency document

The Transparency document associated with this article can be found in online version.

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Author contributions

Study design: M.A., N.M.V., J.J.J., S.M.C.S, J.C.; Generation of zebrafish and HEK293 cell lines: S.M.C.S, F.T., N.L., J.P.W.B., G.vH., S.Z., F.J.T.Z., J.C.; Analytical analysis: M.B., J.G., S.M.C.S, M.A., J.C.; Data interpretation: M.A., S.M.C.S, J.C., N.M.V., J.J.J., F.J.T.Z.; Drafting manuscript: M.A., S.M.C.S, J.C.; Editing manuscript: N.M.V., J.J.J., F.J.T.Z.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbadis.2019.165607.

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