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Pantothenate Kinase Associated Neurodegeneration.

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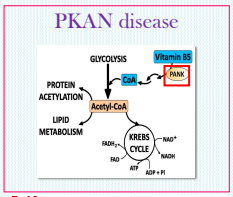
INTRODUCTION

The increased iron deposition is a hallmark of many neurodegenerative diseases, but its pathogenic role is still unclear. A strong link between iron and neurodegeneration is evident in a set of heterogeneous neurological disorders, known as Neurodegeneration with Brain Iron Accumulation (NBIA). The most common form of inherited NBIA is associated with mutations in hPank2 gene (PKAN). Pank2 is the rate limiting enzyme in CoA biosynthesis and its downregulation in mammalian cells leads to perturbation of cellular iron homeostasis. Here we explore Pank2 biological function in Danio Rerio, and propose this system as an important new tool for the study of PKAN disease.

Neurodegeneration with brain iron accumulation (NBIA)

Early onset		Late onset	
Classic PKAN	(Pank2)	Atypical PKAN	(Pank2)
INAD – A.NAD	(PLA2G6)	Neuroferritinopathy	(FtL)
FAHN	(FAH2)	Aceruloplasminemia	(CP)
MPAN	(C19orf12)	Idiopatic NBIA	
BPAN	(WDR45)		
CoPAN	(COASY)		
Kufor Rakeb S.	(ATP13A2)		
Woodhouse-Sakati	(C2orf37)		
Idiopatic NBIA			

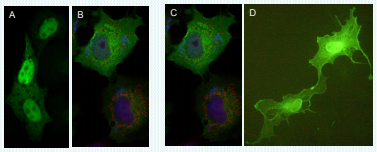


RESULTS

Identification and characterization of *pank2* gene in *Danio rerio*

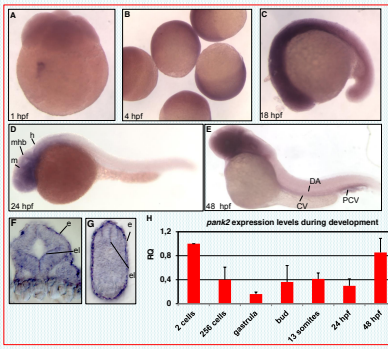
A bioinformatic analysis performed on the latest Zebrafish genome sequence assembly (Zv9) revealed the presence of a single *pank2* gene on chromosome 13, with three different transcripts. The longest one encodes for a putative protein of 437 amino acids with high homology (65%) to the human counterpart. The reciprocal BLAST approach and the sinteny analysis support the hypothesis that the gene represents the *Danio rerio* ortholog of *hPank2*.

Cellular distribution of *Pank2*-Flag protein expressed in HeLa and Cos7 cells



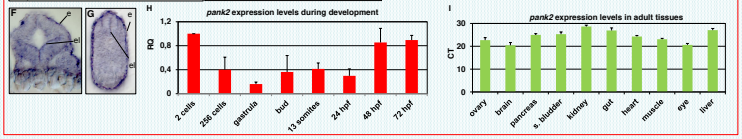
We expressed *pank2* cDNA in HeLa (A,B) and COS7 (C,D) cells and analyzed its distribution by IF. We observed a prevalent diffused staining, indicating a cytosolic distribution of the protein, associated to a nuclear staining. No co-localization with a mitochondrial marker was observed.

Developmental expression of zebrafish *pank2*.

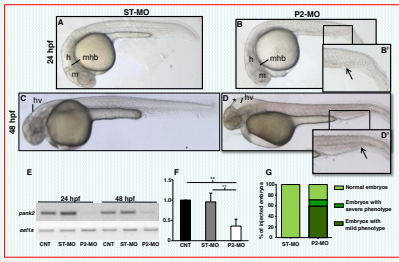


WISH was performed from 1 hpf to 48 hpf with a *pank2* specific, antisense probe. From the "cleavage period" to the somitogenesis the *pank2* signal is diffused (A,B,C). At 24 hpf the transcript is present in defined CNS regions (midbrain, hindbrain and midbrain-hindbrain boundary). At 48 hpf the signal in the brain is still present and also appears in the main vessels and in vessels of the vascular plexus in the tail region (E). Cross sections at the level of the head and trunk of 24 hpf embryos (F,G). Total RNA was extracted from different developmental stages (H) and adult tissues (I) and analysed for *pank2* mRNA by qRT-PCR.

m, midbrain; mhb, midbrain-hindbrain boundary; h, hindbrain; da, dorsal aorta: cv; cardinal vein; pcv, post cardinal vein; e, epidermis; el, ependymal layer.

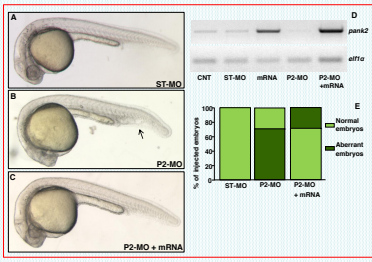


Effects of *pank2* morpholino during zebrafish development.



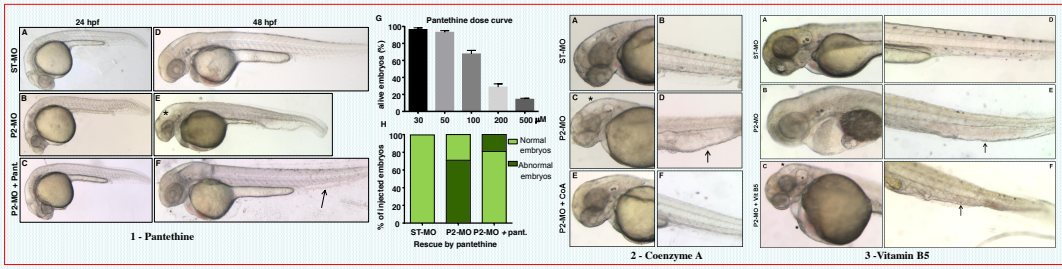
The standard (ST-MO) and the *pank2* morpholino (P2-MO) were micro-injected at the 1/2-cell stage and the phenotype observed at 24 and 48 hpf (A,B,C). At both developmental stages, the morphants showed abnormalities in brain regions, presence of hydrocephalus (*) (D), severe perturbation of the main vessels (dorsal aorta and caudal vein) and the vascular plexus in the tail (arrowhead and magnification B', D'). E) Semi-quantitative RT-PCR analysis of *pank2* transcript levels in non-injected (CNT), ST-MO- and P2-MO-injected embryos at 24 and 48 hpf. F) Densitometric analysis of the RT-PCR products resolved by agarose-gel electrophoresis. G). Quantitative analysis of the phenotype observed in P2-MO injected embryos at 48 hpf. Two different phenotypes could be distinguished: a severe one (12%) with head malformations, presence of hydrocephalus and evident oedema in the caudal plexus, severe defects in anterior-posterior axis and delay in development; a milder phenotype (59%) with not well-defined brain areas, less severe hydrocephalus and edema in caudal plexus.

Rescue by *pank2* mRNA co-injection.



Lateral views of ST-MO- (A), P2-MO- (B) and P2-MO/*pank2* mRNA-injected embryos (C) at 24 hpf. D) Gel electrophoresis of the RT-PCR analysis for *pank2* and *eef1a1l1* mRNAs performed on the described samples. The figure shows a representative experiment out of three with similar results. E) The graph shows the statistical analysis result from the morphological evaluation of embryos from 5 independent micro-injection experiments.

Rescue by pantethine and CoA, but not by vitamin B5

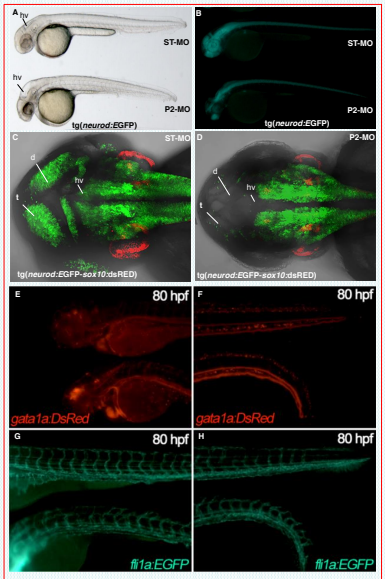


1 - Pantethine

2 - Coenzyme A

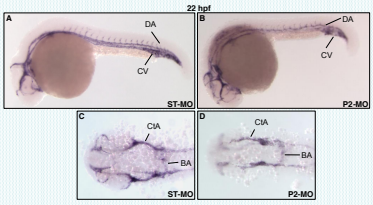
3 -Vitamin B5

Morphological comparison of ST-MO- and P2-MO-injected embryos with P2-MO-injected embryos treated with pantethine 30 µM (1), Coenzyme A 100 µM (2) or Vitamin B5 (3) at 24 and 48 hpf. A high percentage of embryos exposed to pantethine or CoA showed a significant correction of the aberrant phenotype, in particular the edema in the caudal plexus (arrowhead) and the hydrocephalus (asterisk), while Vitamin B5 was completely uneffective.



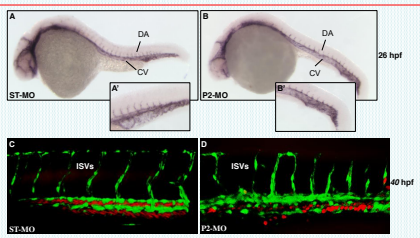
Effects of P2-MO micro-injection in different transgenic lines.

Tg(*neurod:EGFP*) (A,B) Tg(*neurod:EGFP-sox10:mRFP*) (C,D) Tg(*gata1a:DsRed*) (D,E) and Tg(*fli1a:EGFP*) (F,G) transgenic embryos were injected with ST-MO and P2-MO and analysed at 48 (A-D) or 80 hpf (E-H). Abbreviations t, telencephalon; d, diencephalon, hv, hindbrain ventricle.



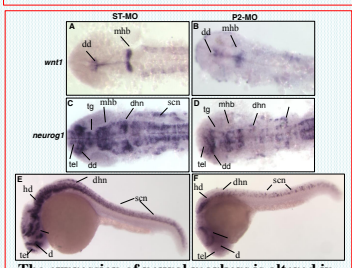
Pank2 down-regulation alters the expression of *VE-cadherin*.

VE-cadherin was analyzed by in *situ* hybridization in control embryos (A,C) and *pank2* morphants (B,D) at 22 hpf. Lateral (A, B) and dorsal views (C, D). Abbreviations: DA, dorsal aorta; CV, cardinal vein; CtA, central arteries; BA, basilar aorta.



Pank2 knock-down affects trunk vessel integrity.

WISH analysis of *fli1a* gene at 26 hpf in ST-MO- and P2-MO-injected embryos (A, A',B, B'). We injected 1 pmole/embryo of ST-MO (C) and P2-MO (D) in Tg(*gata1a:DsRed-fli1a:EGFP*) embryos and analyzed the phenotype by confocal microscopy at 40 hpf. Abbreviations: da, dorsal aorta; cv, caudal vein; isvs, intersegmental vessels.



The expression of neural markers is altered in P2-MO-injected embryos.

The expression of the neural markers *wnt1* (A, B) and *neurog1* (C-F) were analyzed by WISH at 24 hpf. A-D dorsal views, E and F, lateral views. Abbreviations: dd, dorsal diencephalon; tel, telencephalon; tg, tegmentum; mhb, midbrain-hindbrain boundary; hb, hindbrain; dnn, dorsal hindbrain neurons; scn, spinal cord neurons.

CONCLUSIONS

The *danio rerio pank2* protein shows 65% identity with the human ortholog. The qRT-PCR analysis on total RNA from embryos and adult tissues showed that the expression of *pank2* transcript is detected in the embryos from the early stages to 72 hpf. The brain is the tissue with the highest expression level of *pank2* transcript. The whole-mount *in situ hybridization* technique confirmed the qRT-PCR results, showing high expression in different brain structures, in the main vessels and in the venous plexus. The microinjection of a *pank2*-specific morpholino resulted in a clear-cut phenotype, with perturbation of the CNS structures and the vascular system development, suggesting the relevance of *pank2* expression for the normal nervous and vascular developmental process in zebrafish. Both the co-injection of *pank2* mRNA and the addiction of pantethine 30 µM at the gastrula developmental stage restored the wild type phenotype with high efficiency. The effects induced in the CNS and the vascular structures were characterized by WISH with different neuronal and vascular markers and by injecting the morpholino in different transgenic lines. The results indicated a clear effect on the development of a subset of brain regions in the forebrain, where also the nuclei corresponding to the human globus pallidum localize. The vascular arborization was also drastically perturbed, with severe fenestration of the main vessels and reduced connections of the inter-somitic vessels. Altogether the data indicate that the transient down-regulation of *pank2* gene expression in zebrafish represents an interesting model of PKAN disease, potentially amenable for high-throughput screening of molecules with therapeutic potential.