α3Na+/K+-ATPase deficiency causes brain ventricle dilation and abrupt embryonic motility in zebrafish*

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Background: The Na+/K+-ATPase maintains Na+/K+ gradients across the plasma membrane, essential for cellular functions.

Results: Zebrafish deficient of α3Na+/K+-ATPase display abnormal motility and brain ventricle dilation.

Conclusion: Zebrafish α3Na+/K+-ATPase is CNS-specific and required for brain ventricle maintenance and embryonic motility.

Significance: This is the first study assessing α3Na+/K+-ATPase in brain development and embryonic motility.

SUMMARY

Na+/K+-ATPases are transmembrane ion pumps that maintain ion gradients across the basolateral plasma membrane in all animal cells to facilitate essential biological functions. Mutations in the Na+/K+-ATPase alpha3 subunit gene (ATP1A3) cause rapid-onset dystonia-parkinsonism (RDP), a rare movement disorder characterized by sudden onset of dystonic spasms and slow movements. In the brain, ATP1A3 is principally expressed in neurons. In zebrafish, the transcripts of the two ATP1A3 orthologs, Atp1a3a and Atp1a3b, show distinct expression in the brain. Surprisingly, targeted knockdown of either Atp1a3a or Atp1a3b leads to brain ventricle dilation, a likely consequence of ion imbalances across the plasma membrane that cause accumulation of cerebrospinal fluid in the ventricle. The brain ventricle dilation is accompanied by a depolarization of spinal Rohon-Beard neurons in Atp1a3a knockdown embryos, suggesting impaired neuronal excitability. This is further supported by Atp1a3a or Atp1a3b knockdown results where altered responses to tactile stimuli as well as abnormal motility were observed. Finally, proteomic analysis identified several protein candidates highlighting proteome changes associated with the knockdown of Atp1a3a or Atp1a3b. Our data thus strongly supports the role of α3Na+/K+-ATPase in zebrafish motility and brain development - associating for the first time the α3Na+/K+-ATPase deficiency with brain ventricle dilation.

The Na+/K+-ATPase is essential for maintaining Na+ and K+ gradients across the plasma membrane, required for many cellular functions,
e.g., regulation of cell volume, pH, Na\(^+\)-coupled secondary transport of molecules and neurotransmitters, and the excitability of muscle and neuronal cells (1,2). In mammals, the four \(\alpha\) (\(\alpha_1, \alpha_2, \alpha_3, \alpha_4\)) isoforms display distinct tissue-specific expression patterns (2,3). The \(\alpha_3\) isoform (ATP1\(\alpha_3\)) is expressed in brain, eye, ear, muscle, cartilage, uterus, placenta, and heart (4-7). In the brain, the ATP1\(\alpha_3\) subunit is present exclusively in neurons (8). Mutations in the gene cause the neurological disorder rapid-onset dystonia-parkinsonism (RDP) (9,10), a rare movement disorder with abrupt onset and rapid (hours to weeks) development of dystonia parkinsonism, primarily bradykinesia and postural instability (10,11).

Besides ion pump roles, Na\(^+\)/K\(^+\)-ATPases also serve as signal transducers, modulating synaptic plasticity, e.g., inducing dendritic growth in cortical neurons (12). Evidence for neuronal roles of ATP1\(\alpha_3\) was previously found in both human and rat dorsal root ganglia (DRG), and also observed in rat embryos (E21) (13).

Currently, two different genetically modified mouse models targeting the ATP1\(\alpha_3\) gene exist: \(\alpha_3\)^{KI\(\alpha_3\)} (14) and \(\alpha_3\)^{K180N\(\alpha_3\)} (Myshkin (Myk)) (15). These mice display learning/memory deficits (14), epilepsy/seizures (15), stress-induced motor symptoms (16), anxious phenotype and depression-like behavior (17). However, they do not fully capture the human RDP symptoms, and the pathology of RDP remains unsolved.

Recently it became more evident that Danio rerio (zebrafish) is a valuable model for investigating Na\(^+\)/K\(^+\)-ATPase functions (18,19). We employed zebrafish, with its advantageous properties, e.g., external development with optical clarity, small size, short generation time (2-3 months) and high fecundity (20,21) to further study the role of ATP1\(\alpha_3\) in early neuronal functions. In a comparison of the zebrafish brain structure with human, the gross architecture of many zebrafish brain areas, e.g., retina, olfactory bulb, hypothalamus, cerebellum, and spinal cord, is similar to that of humans, although there exist some differences between teleosts and mammals (22). Moreover, zebrafish enables several behavioral and drug tests, hence is relevant for many disease-related studies.

Zebrafish have two ATP1\(\alpha_3\) orthologs, ATP1a3a and ATP1a3b (23). Surprisingly, knockdown (KD) of ATP1a3a or ATP1a3b results in severe brain ventricle dilation in contrast to previous data where KD of Atp1a1 caused reduced brain ventricle inflation, thus supporting the role of Na\(^+\)/K\(^+\)-ATPase in brain ventricle development. The brain ventricle dilation in ATP1a3a/b-KD embryos was accompanied by a ~20 mV depolarization of the spinal Rohon-Beard (RB) neuron resting membrane potential (RMP), suggesting compromised functions in neurons as a consequence of ATP1a3a KD-generated defects in ion homeostasis.

In support of this, morphant embryos further displayed both abnormal touch response and spontaneous movements. To identify additional neuronal functions that may depend on correct ion homeostasis for normal performance, we used a proteomic approach. Interestingly, this revealed several proteins, including cytoskeletal, ion binding, muscle associated proteins, etc., with expression levels that were affected by ATP1a3a or ATP1a3b KD.

This is the first detailed report of \(\alpha_3\)Na\(^+\)/K\(^+\)-ATPases in zebrafish and on the basis of conservation of zebrafish and mammalian \(\alpha_3\)Na\(^+\)/K\(^+\)-ATPase expression and functions. Zebrafish can serve as an advantageous model for analysis of brain ventricular volume maintenance and embryonic motility, both related to ion homeostasis.

**EXPERIMENTAL PROCEDURES**

**Animals**—Tübingen (TU) zebrafish strain (Nüsslein-Volhard lab, Max Planck Institute, Tübingen, Germany) and Tg(gfap:GFP) transgenic line (Zebrafish International Resource Center, University of Oregon, USA) of either sex were used in the experiments. Embryo maintenance and staging were performed as previously described (24,25). To conserve optical clarity, embryos were raised in the presence of 0.2 mM 1-phenyl-2-thiourea (PTU) (24).

Reverse transcription (RT) and qRT-PCR—qRT-PCR protocol was performed on cDNAs from embryos at corresponding stages (1k-cell, 50% epiboly, 75% epiboly, 6-somite, prim-6, prim-22, pec fin and adult (one male + one female)) as previously described (26). Pearl Primer software (27) and Roche assay design were used to design the primers (Table S2) to detect transcripts of ATP1a3a (accession number: NM_131684.2), ATP1a3b (accession number: NM_131685.2), Actb2 (accession number: NM_181601.3); and Bhmt, Ckma, Ckmb, Gamt, Gipb, Krt4, Krt5, Mhyz2, Ndpkz2, Pgam2, Pvalb9, Zgc:91930 and 113d7.4, accession numbers of which are provided in Table 1. Generated PCR product sizes and iden-
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Embryos were embedded and oriented in 5% agar, so they could be cut transversely following dehydrating in ascending alcohol solutions (70% x2, 96% x2 and 99% x2), infiltration and embedding in glycolmethacrylate (Technovit 7100). Using a Microm 355, 20-μm-thick sections were cut and every tenth section was taken by systematic sampling.

Whole-mount embryos and sections were observed using an inverted microscope, Olympus IX71.

MO-mediated knockdown of Atp1a3a or Atp1a3b and mRNA rescue– MOs (Gene Tools, LLC) for microinjection were diluted in distilled water and microinjected into embryos at 1-4 cell stage. Translation blocking MOs: α 3a-MO (5’TCTTCTTCAGTCTGTCAACCGGTGTT-3’) (12 ng) and α 3b-MO (5’-AGAGTGAAGAAAGTACAGCCT-3’) (3 ng); the splicing blocking MOs: α 3a-SP-MO (5’TCCACCTGAGCAATGACACCCAAC-3’) (11 ng), targeting intron 6- exon 7 boundary, and α 3b-SP-MO (5’-AGTGCCGTGAAACAAAGCATTTT-3’) (3 ng), targeting intron 7-exon 8 boundary; the standard control MO (std-MO: 5’-CCTTTTACCTCAGTTACAATTATA-3’) (3, 12 ng); and the p53-MO (5’-GCGCCATTGCTTTGCAAGAATTG-3’) (4 ng) were injected at indicated amounts. Embryos were grown for 60 h in embryo medium with 0.003% PTU and observed under inverted microscope, Olympus IX71. In order to visualize zebrafish brain ventricles, rhodamine-conjugated dextran was injected into the brain ventricle at 48 hpf as previously described (30), and embryos were subsequently imaged under bright field and fluorescent light (Olympus IX71).

The template for Atp1a3a and Atp1a3b mRNA was generated by PCR on pTZ57R vector harbouring Atp1a3a probe coding sequence, for Atp1a3a; and by RT-PCR on adult zebrafish RNA, for Atp1a3b, introducing T7 priming site in both scenarios. Probes were Digoxigenin (DIG)-labeled during synthesis from purified PCR products by using the DIG RNA Labeling Mix (Roche) and T7 Polymerase (Roche) following the manufacturer’s protocol. Embryos were fixed overnight in freshly prepared 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS, pH 7.4) and kept in MeOH in -20°C. To detect Atp1a3a and Atp1a3b transcripts, whole-mount in situ hybridization was performed on embryos at 60 hpf, as previously described (29) with minor modifications.

In order to investigate SP-MO-mediated KD, we initially performed RT-PCR using primers enclosing the presumably extruded exons. This clearly indicated that both SP-MOs caused a KD via nonsense-mediated mRNA decay (data not shown). The quantitation of KD levels was performed by qRT-PCR on 1st strand cDNA generated from ~30 WT, std-MO (5.5, 11, 3 ng), α 3a-SP-MO (5.5, 11 ng) and α 3b-SP-MO (1.5, 3 ng) injected embryos.

Efficiency of each primer pair used in qRT-PCR was detected to be approximately 100%. Transcription levels were quantified using the relative quantification method based on comparative threshold cycle values (Ct).

Cloning of zebrafish Atp1a3a and Atp1a3b cDNA– Total RNA was isolated from a male and a female zebrafish by TRIzol® method (Invitrogen) following the manufacturer’s instructions (29) with minor modifications. The template for RT-PCR was detected to be approximately 100%.

In situ hybridization and sectioning– Anti-sense and sense RNA probe templates were generated by PCR on pTZ57R vector harboring Atp1a3a probe coding sequence, for Atp1a3a; and by RT-PCR on adult zebrafish RNA, for Atp1a3b, introducing T7 priming site in both scenarios. Probes were Digoxigenin (DIG)-labeled during synthesis from purified PCR products by using the DIG RNA Labeling Mix (Roche) and T7 Polymerase (Roche) following the manufacturer’s protocol. Embryos were fixed overnight in freshly prepared 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS, pH 7.4) and kept in MeOH in -20°C. To detect Atp1a3a and Atp1a3b transcripts, whole-mount in situ hybridization was performed on embryos at 60 hpf, as previously described (29) with minor modifications.

Transcription levels were quantified using the relative quantification method based on comparative threshold cycle values (Ct).
ventricle dilation (+, slight/no; ++, moderate; ++++, severe) (Fig. 2D, lower panel) and quantified as percentages of the total number of embryos. Experiments were repeated at least three times and data is presented as mean±s.d.

**Atp1a3a RNA in situ hybridization and TH immunostaining**—WT TU zebrafish were raised in embryo medium with PTU. RNA in situ hybridization was adapted from Schulte-Merker et al. (31) and immunostaining was subsequently performed as previously described (29) with minor modifications. In situ hybridization was applied on non-injected embryos using Atp1a3a antisense riboprobe and the final labeling was maintained by FastRed tablets (Sigma). Stained embryos were fixed in 4% PFA, blocked in 10% HIGS/PBST and incubated in anti-TH primary antibody (Millipore, UK) at 1:200 final concentration overnight at 4°C. Following the washes, embryos were incubated in goat anti-mouse secondary antibody conjugated to Alexa 488 at 1:1000 final concentration, and incubated in anti-TH primary antibody (Millipore, Sunnyvale, CA). Recordings were performed at room temperature, using bath solution (in mM: 125 NaCl, 3 KCl, 10 CaCl2, and 5 HEPES; pH 7.4). RMP recordings were obtained from RB cells of these embryos together with WT and std-MO injected control embryos using patch electrodes and an Axopatch-200B amplifier (Axon Instruments, Molecular Devices, Sunnyvale, CA). Recordings were performed at 4°C.

**Quantification of DA neurons**—Tyrosine hydroxylase (Th) antisense riboprobe was generated as Atp1a3b riboprobe, described above. In situ hybridization protocol to detect Th mRNA expression, applying labeling via FastRed tablets, was adapted from Schulte-Merker et al. (31), and performed on WT, α3a-SP-MO-mediated Atp1a3a KD and Atp1a3a mRNA rescued Atp1a3a-KD embryos. Embryos were observed under inverted microscope, Olympus IX71. Bright field images of Th riboprobe hybridized WT (N=10) and α3a-SP-MO mediated Atp1a3a-KD embryos (N=10), were quantified in terms of DA neuron content. Due to the extent of the brain ventricle dilation, it was not possible to quantify the number of DA-neurons by single-cell count, and thus, total staining of labeled DA neurons in these embryos were quantified using a semi-quantitative open source image analysis software ImageJ (US National Institutes of Health, Bethesda, MD, USA). Mean integrated density±s.d. were plotted.

**Whole-cell patch-clamp electrophysiology**—Atp1a3a–Atp1a3a-KD and control embryos at 48 hpf were sacrificed, skinned and mounted dorsally to access to RB cells as previously described (32).

Atp1a3a-KD embryos were grouped into two according to brain ventricle dilation severity; ones with severe (+++) and slight/no (+) dilation. Electrodes were made to a tip resistance of 2.6-3.7 MΩ using a P-97 microelectrode puller (Sutter Instruments, Novato, CA) and filled with intracellular pipette solution (in mM: 135 KCl, 10 EGTA, and 10 HEPES; pH 7.4). RMP recordings were obtained from RB cells of these embryos together with WT and std-MO injected control embryos using patch electrodes and an Axopatch-200B amplifier (Axon Instruments, Molecular Devices, Sunnyvale, CA). Recordings were performed at room temperature, using bath solution (in mM: 125 NaCl, 3 KCl, 10 CaCl2, and 5 HEPES; pH 7.4) under inverted microscope Zeiss Axioskop (Germany). Data is presented as mean±s.e.m.

**Touch response**—Mechanosensory stimulation was delivered to the embryo trunk with a needle. WT, std-MO, α3a-MO and α3b-MO injected embryos with brain ventricle dilation phenotype at 60 hpf were recorded under an Olympus IX71 microscope at 25 frames/sec. Experiments were independently repeated at least three times.

**Proteomics analysis by isobaric tags for relative and absolute quantitation (iTRAQ)**—30-50 of std-MO, α3a-MO, α3b-MO injected embryos, 60 hpf, were collected in eppendorf tubes, were lysed in lysis buffer (0.02 M Tris, 0.137 M NaCl, 1% NP-40, 10% Glycerol, 1 mM PMSF, 1X complete Protease inhibitor cocktail tablet (Roche)) with the help of a homogenizor. Cell lysate was analyzed by iTRAQ LC-MS/MS as previously described (33). Briefly, the cell lysate was in-solution digested using trypsin; labeled with the iTRAQ (reporter ions m/z 114 and 115) followed by fractionation of the peptide mixture using hydrophilic interaction chromatography and LC-MS/MS analysis of the peptide fractions. Raw data files were processed using the Proteome Discoverer software (version 1.3) integrated with the MASCOT database search program (version 2.2.3). Data were searched against the nrDB database restricted to D. rerio.

**RESULTS**

Specific expression of Atp1a3a and Atp1a3b transcripts in the developing zebrafish brain—As an initial step to understand the roles of the α3Na+/K+-ATPases, we assessed expression of Atp1a3a and Atp1a3b mRNA by qRT-PCR. We detected the Atp1a3a transcript at the 1000-cell (1k cell; 3 hpf) and 50% epiboly (5.3 hpf) stages.
Thereafter it declined and was barely detectable by 75% epiboly (8 hpf) and at the 6-somite (12 hpf) stage, suggesting that these early-detected transcripts were provided maternally. *Atp1a3a* reappeared at the prim-6 (25 hpf) and prim-22 (35 hpf) stages. The highest expression of *Atp1a3a* occurred at the pec fin stage (60 hpf), which was almost six-fold higher than the relative expression of the *Atp1a3a* transcript in adult zebrafish (Fig. 1A). In contrast, the *Atp1a3b* mRNA was barely detectable from the 1000-cell to the 6-somite stage (Fig. 1A), suggesting lack of maternal *Atp1a3b* contribution. Similar to *Atp1a3a*, the *Atp1a3b* transcript appeared at prim-6 and onwards, and also showed a marked increase at the pec fin stage (Fig. 1A). Further experiments were performed at 48-60 hpf, due to the notable expression of both isoforms at these stages and the fact that the embryonic CNS is well structured by these stages. At these time points, the MO is still effective.

To determine whether of *Atp1a3a* and *Atp1a3b* transcripts were present in the central nervous system (CNS), we then performed in situ hybridization. The lack of antibody that detects the zebrafish α3 isoforms did not allow us to directly test protein levels, however we assume that the detected transcripts give rise to protein products and represent active Na⁺/K⁺-ATPase pumps in the plasma membrane of the cell.

We showed that *Atp1a3a* transcripts localize to several CNS structures, including the epiphysis, tegmentum, tectum, cerebellum, cranial ganglia, hindbrain, and spinal cord (Fig. 1B). A dorsal view of the head shows a dense accumulation of the *Atp1a3a* transcript throughout the brain. Transverse rostral sections illustrate in more detail *Atp1a3a* expression in several brain structures (Fig. 1B, section I). More caudal sections show *Atp1a3a* expression in different CNS regions including the cranial ganglia (Fig. 1B, section II) and spinal cord (Fig. 1B, sections III and IV).

In contrast to the widespread expression of *Atp1a3a*, the *Atp1a3b* transcript was detected in a more restricted but overlapping set of brain structures such as epiphysis, tegmentum, cranial ganglia, hindbrain and anterior spinal cord, evident in both lateral and dorsal views (Fig. 1C). Transverse sections show *Atp1a3b* expression in the hindbrain and cranial ganglia (Fig. 1C, sections I and II), in the rostral (Fig. 1C, section III) but not the caudal (Fig. 1C, section IV) spinal cord.

**Knockdown of Atp1a3a causes brain ventricle dilation**—To test for a role of the α3aNa⁺/K⁺-ATPase in zebrafish development, we used an antisense MO oligonucleotide mediated KD approach. The translation-blocking MOs cause the phenotypes of p53-dependent effects, e.g. apoptosis, did not affect on the phenotype (Fig. 2A) suggesting that the protein function is significantly reduced.

The efficiency of *Atp1a3a*-KD induced by α3a-SP-MO was determined by qRT-PCR, demonstrating up to 62% reduction in the *Atp1a3a* transcript with increasing MO concentration (Fig. 2C).

Marked brain ventricle dilation was observed upon KD of *Atp1a3a* using either translation- (α3a-MO), or splice-blocking (α3a-SP-MO) MOs (Fig. 2A), and was further visualized by rhodamine-conjugated dextran injection into zebrafish brain ventricles (Fig. 2B). Use of the transgenic line, Tg(gfap:GFP), that expresses green fluorescent protein (GFP) in astrocytes provided an overall view of the CNS structure. A pressure to the brain structures from the ventricle dilation was evident upon *Atp1a3a* KD (Fig. 2B). Injection of α3a-SP-MO led to severe, moderate or slight/no brain ventricle dilation in 54%, 36% or 11% of embryos, respectively (Fig. 2D), while std-MO injected embryos appeared morphologically normal (Fig. 2A).

To exclude that the observed brain ventricle dilation was caused by nonspecific MO-induced activation of p53-dependent apoptosis (34), control experiments were carried out: Co-injection of p53-MO with any of the MOs did not have an effect on the phenotype (Fig. 2A) suggesting that p53-dependent effects, e.g. apoptosis, did not cause the phenotypes of *Atp1a3a* KD. To further confirm the specificity of the *Atp1a3a*-KD phenotypes, embryos were co-injected with in vitro-synthesized *Atp1a3a* mRNA. In the rescued embryos, the extent of the brain ventricle dilation was reduced compared to *Atp1a3a*-KD embryos, and severe, moderate and slight/no ventricle dilation was present in 21%, 29% and 50%, respectively (Fig. 2D). Taken together, these findings suggest that *Atp1a3a* KD had specific effects that led to the brain ventricle dilation in α3a and α3a-SP MO-injected embryos.
Atplab deficient embryos phenocopy the Atplab deficient embryos--We used a similar approach to assess the developmental role of the other ATP1A3 ortholog, Atplab. qRT-PCR analysis showed that the α3b-SP-MO led to reductions in transcript levels of 22% and 66% when injected in amounts of 1.5 ng and 3 ng, respectively (Fig. 3C). Moreover, Atplab-KD embryos showed brain ventricle dilation phenotype similar to those produced by Atplab-KD embryos (Fig. 3A,B). Atplab KD caused severe brain ventricle dilation in 59%, moderate in 27%, and no extraordinary ventricle dilation in 14% of the embryos and std-MO injected embryos appeared morphologically normal (Fig. 3A,D). Co-injections of p53-MO ruled out the activation of p53-dependent apoptosis in Atplab-KD embryos (Fig. 3A). Upon co-injection with in vitro transcribed Atplab mRNA, severe brain ventricle dilation was observed in 16%, moderate in 24%, and no ventricle dilation in 61% of the embryos (Fig. 3D). Importantly, the brain ventricle dilation of Atplab-KD embryos was not rescued by co-injection of Atplab mRNA, and conversely, Atplab-KD embryos were not rescued by co-injection of Atplab mRNA (Fig. 3E).

The observation of similar phenotypes upon the use of two distinct MOs (Figs 2A,3A), and the fact that the nonsense-mediated mRNA decay of the targeted transcript by SP-MOs could be observed in a concentration-correlated manner (Figs 2C,3C), together verified the specificity of the MOs used in this study. In addition, our observations of consistent brain ventricle dilation phenotypes, even after p53-MO co-injection with each MO (Figs 2A,3A), and importantly, the significant rescue of the phenotypes by injection of the mRNA of the knocked down gene (Figs 2D,3D), further confirmed the specificity. It is important to note that full rescue of the brain ventricle dilation phenotype was not accomplished most likely due to limited stability of the injected exogenous mRNA in the cell.

Atplab-KD leads to depolarization of the Rohon-Beard neurons resting membrane potential--RB cells are mechanosensory neurons localized in the dorsal spinal cord (35,36), and are involved in response to touch, a behavior that involves both neuronal and muscular components. RB neurons are within the domain of Atplab expression (Fig. 1B) and accessible due to their superficial localization. Combining the advantages of Atplab being expressed in RB neurons and the accessibility of these cells, we measured electrophysiological changes at the cellular level in RB neurons of Atplab-KD embryos. The RMP of RB neurons was recorded using whole-cell patch-clamp. The RMP of RB neurons in Atplab-KD embryos displaying severe brain ventricle dilation was significantly (p<0.01) more depolarized (mean RMP: -46.6 ± 5.1 mV) compared to the RMP from slight/no brain ventricle dilation-displaying Atplab-KD embryos (mean RMP: -70.4 ± 2.9 mV), which were comparable (p>0.05) to the control groups, non-injected (mean RMP: -72.1 ± 1.7 mV) and std-MO injected (mean RMP: -65.2 ± 3.1 mV) embryos (Fig. 4A).

A schematic representation illustrates the consequence of the depolarized RMP in RB neurons in an Atplab-KD embryo (Fig. 4B).

Atplab-αa and Atplab-KD embryos differ in their mechanosensory responses--The onset of Atplab-αa and Atplab expression coincides with the ability of the embryo to respond to tactile stimulation at around 27 hpf (prim-6 stage), where a marked increase in the expression of both isoforms was detected (Fig. 1A). Considering that RB cells mediate touch sensitivity for the embryo, and that KD of Atplab depolarized the RB RMP, we tested for effects on tactile sensitivity. The RB-mediated touch response is assayed by applying tactile stimuli to the trunk of a motile embryo (37). Whereas, both Atplab-αa- and Atplab-KD embryos display abnormal spontaneous motility (data not shown), they are motile and suitable for assessment of tactile sensitivity.

At 60 hpf, the control embryos, non-injected (Movie 1) and std-MO injected (Movie 2), responded by burst swimming as expected. Atplab-KD embryos (Movie 3) typically responded quickly to tactile stimulation but with a brief escape and circling movements that in one instance culminated in convulsion (top). Atplab KD also had a range of effects, some of which were similar to and others different from those produced by Atplab-KD (Movie 4). The most consistent difference was that Atplab-KD embryos responded with a delay in contrast to the quick response of Atplab-KD embryos. Similar to Atplab-αa-KD embryos, Atplab-KD embryos also displayed brief distance recoils and, in one instance, convulsion (right). Successive frame shots from touch response movies (movie 1, 3 and 4) provide an overview of the embryonic movements and allow comparisons between the different experimental groups (Fig. 5). Taken together, the data indicate...
that Atp1a3a or Atp1a3b KD impair but do not prevent embryonic motility. In addition, the embryos retain the ability to respond to touch but, given the motility defects, it was difficult to assess whether there was any reduction in touch sensitivity, although this is indicated by RMP alterations in RB neurons in Atp1a3a-KD and by delayed response in Atp1a3b-KD embryos.

Atp1a3a KD leads to disorganization of the brain but no loss of dopaminergic neurons—A subset of dystonia subtypes respond to L-DOPA treatments that target dopaminergic (DA) neurons in the substantia nigra (38). However, RDP patients do not respond to L-DOPA treatment. To assess whether zebrafish α3 isoforms differ in their association to DA neurons compared to their mammalian counterparts, we questioned the presence of zebrafish α3 isoforms in DA neurons. Atp1a3a co-localized with the DA neuron marker, TH (Fig. 6A), a marker previously used to identify DA neurons in zebrafish (39). Confocal images highlight the classical arrangement of TH positive cells in the diencephalon, region that shows widespread expression of Atp1a3a mRNA (Fig. 6A). The expression pattern of Atp1a3b (Fig. 1C) does not suggest co-expression of this isoform with DA neurons.

We then tested whether Atp1a3a KD affected DA neurons by comparing the distribution of Th transcripts in WT, α3-SP-MO-mediated Atp1a3a KD, and mRNA rescued Atp1a3a-KD embryos (Fig. 6B). Whereas there were some slight differences in the pattern and intensity of the Th mRNA signal (Fig. 6B), quantitative analysis showed no significant difference (Fig. 6C). The variations in Th expression profile observed in Atp1a3a-KD zebrafish correlate with the extent of brain ventricle dilation and the consequent spatial reorganization in the brain, but not with loss of DA neurons. These results support the view that Atp1a3a KD does not lead to DA neuron degeneration.

Several proteins are up/down-regulated when either Atp1a3a or Atp1a3b is knocked down—In order to investigate the cellular protein networks that potentially involve α3Na+/K+-ATPases, we carried out proteomics analyses. Candidate proteins affected in Atp1a3a- or Atp1a3b-KD embryos were identified using isobaric tags for relative and absolute quantitation (iTRAQ), and listed together with their known tissue associations (Table S1). Interestingly, several candidates are of relevance to Na+/K+-ATPase and Atp1a3a- or Atp1a3b-KD phenotypes (Table 1), but only selected candidates are discussed below. It is interesting to note that cytoskeletal and muscle associated proteins appear to be regulated by both α3 isoforms. In contrast, the level of ion binding proteins and proteins involved in phosphate metabolism appear to depend on the α3b isoform.

We performed qRT-PCR to verify selected proteomic candidates, as antibodies toward all of these zebrafish proteins were not available. We found that these transcripts were also down-regulated in Atp1a3a or Atp1a3b-KD embryos, supporting the proteomics data at the transcript level (Fig. 7A,B).

DISCUSSION

In this study, we analyzed zebrafish α3Na+/K+-ATPases to gain insight on specific functions of this particular ion pump and how we might use this model to elucidate the functions of the Na+/K+-ATPase in brain development. The Na+/K+-ATPase has a well-known role as a modulator of membrane potential in neurons and is essential for generating an action potential. Consistent with the mammalian ATP1α3 expression, both ATP1A3 zebrafish orthologs are expressed primarily in the brain. Interestingly, the expression of the Atp1a3a mRNA is widely distributed in the brain, in contrast to a more restricted expression of the Atp1a3b mRNA, in line with the expression data available at The Zebrafish Model Organism Database (zfin.org). The Atp1a3a transcript was also abundant in the spinal cord and, to a lesser extent, in the heart. The latter observation is consistent with previous reports demonstrating ATP1α3 expression in the neonatal, but not adult, rat heart (40,41).

Despite the distinct expression profiles of Atp1a3a and Atp1a3b transcripts, both Atp1a3a and Atp1a3b deficiencies cause brain ventricle dilation - rather than cellular swelling, indicating an altered fluid and electrolyte balance. Possibly, the subset of ventral CNS regions that express both Atp1a3a and Atp1a3b involves a pathological pathway that results in this phenotype A functional Na+/K+-ATPase, in a complex with aquaporins and glutamate transporters, is important for maintaining water and ion balance in the brain (42). Interestingly, Na+/K+-ATPase, in particular Atp1a1, was previously demonstrated to be required for the
zebrafish brain ventricle development (19,43). However, our data suggest that Atp1a3a and Atp1a3b KD does not inhibit ventricle inflation, but the consequent phenotype is connected to dilated ventricles as a result of cerebrospinal fluid (CSF) accumulation. An increase or a decrease in the CSF volume can be pathological throughout life (44) and the Na+/K+-ATPase may serve to sense the ventricular volume in a homeostatic role. Consequently, even a slight disruption of Na+/K+-ATPase subunits could lead to significant changes in CSF volume. Overall, these data indicate a role of α3Na+/K+-ATPase in brain ventricle volume maintenance though its ion pump function. CSF composition is vital to brain health. The protein function of the α3Na+/K+-ATPase in neurons is likely connected to the extrusion of Na+ to the extracellular space coupled to the uptake of K+ into the intracellular cytoplasm. This exchange of Na+/K+ ions generates an electrochemical gradient, which, in turn, facilitates ion transports of e.g Cl-, HCO3- and H2O, required to regulate CSF volume (45). Interestingly, a recent study showed that increases in aquaporin-1 (AQP1) and cation chloride transporters (Na+-K+-2Cl- cotransporter 1 (NKCC1)) expression under hyposmotic stress may be one of the molecular mechanisms underlying the pathophysiology of acute hyponatremia, by increasing water transport across the blood-CSF barrier (46).

It is very interesting that the distinctively expressed α3 isoforms both result in brain ventricle dilation when knocked down, and moreover, that they are not able to cross rescue. This indicates that at some level, the ion pump function must be the major mechanism behind our observations despite the different proteomic changes when knocked down. Furthermore, we do not know at this stage to what extent other Na+/K+-ATPase isoforms are active in the cells expressing these α3 isoforms.

Modeling human diseases in other organisms requires complex analysis. Of particular relevance for this study is the fact that the water environment hosting zebrafish differs from other environments and thus requires other mechanisms for ion homeostasis to maintain osmolarity. However, we believe it is not so extraordinary to observe a brain ventricle dilation phenotype in the Atp1a3-KD zebrafish, although this is not a manifestation observed in RDP patients harboring mutations in the ATP1A3 gene (47). Thus, we cannot rule out that the brain ventricle dilation is specific to zebrafish, despite the conserved functions of the Na+/K+-ATPase. The CSF fills the ventricle of the brain, the composition of the CSF influences neuronal activity, and it serves as a drainage pathway for the brain. The latter function might be significantly different in water-living animals. In summary, it appears that the α3Na+/K+-ATPase is a novel regulator of brain ventricle volume, at least in zebrafish.

Minor changes in ion composition in the extracellular and intracellular compartments of the brain can significantly affect neuronal function, which relies on precise ion gradients across their plasma membranes to trigger changes in membrane potentials underlying action potential generation and propagation. Therefore, ion and water transport in the brain are tightly regulated (48). The most studied role of the α3 isoform is its neuronal function, and failure of the Na+/K+-ATPase to maintain Na+ and K+ gradients leads to a decrease in both the RMP and the action potential, and to altered neuronal excitability in several different neurons, such as rat hippocampus dentate interneurons, pyramidal and purkinje neurons (49). Consistent with this, we found that RB neurons had depolarized RMPs when Atp1a3a was knocked down. This result is important and additionally serves to strongly support the specificity of the Atp1a3-KD phenotypes. The change in the electrophysiological state of the RB neurons is thus predicted to alter the neuronal excitability, although future in vivo measurements of excitability are required to address this. Our data strongly supports a neuronal function for α3 isoforms in zebrafish. We believe that it is important to keep in mind that the RMP recording is performed in RB neurons and the ventricle dilation is observed in the brain. We do not yet know the contributions from other Na+/K+-ATPase isoforms in these cell populations, and this could certainly explain why we could not demonstrate an altered RMP in the slight (if any) brain ventricle dilation-displaying embryos.

The production of any touch response in zebrafish can be divided into several steps from sensory perception to muscle activation. The mechanosensory neurons, in this case RB neurons, sense touch stimuli. Once triggered by sensory input, interneuronal networks located in the hindbrain and spinal cord produce the appropriate motor rhythm (50). Depolarization in RB neurons,
indicated abnormality in the sensory component of this type of behavior, although further experiments are needed to distinguish all the contributors of this phenotype. It is intriguing since the motor deficits observed in α3−/−KO mice were shown to have neuronal origin (16). Furthermore, inefficient central sensory-motor processing has been questioned to be a possible causative mechanism for dystonia, and numerous clinical phenomena suggest the primary involvement of the somatosensory system in this disorder (51,52).

Intriguingly, a recent study reported Na+/K+-ATPase as an important player in locomotor behavior of frog tadpoles (53), and of Drosophila larvae (54). Also, in spinal network of neonatal rats, blocking of Na+/K+ pump activity disrupts rhythmic bursting of lumbar motor neurons (55,56). Moreover, the pump function of Na+/K+ -ATPase was recognized as a mechanism to gate sensory information entering the spinal cord, where it alters neuronal excitability (57). By a similar mechanism, the changes in neuronal excitability might account for the impaired spontaneous motility of the Atp1a3a- and Atp1a3b-KD embryos.

Treatment of RDP patients with L-DOPA has no effect, and the DA re-uptake sites appear normal in such patients (58). Although studies in mouse brain did not detect the α3 isoform in DA neurons of substantia nigra (8), it is interesting that another subunit, the beta subunit, ATP1b1, is down-regulated in DA neurons of patients with Parkinson’s disease (59). We detected Atp1a3a expression in zebrafish TH-positive cells. In line with this, a recent study identified Atp1a3a as a target of a cardiac glycoside (a Na+/K+ -ATPase inhibitor; Neriifolin), which impairs DA neuronal survival (60). However, our results show that Atp1a3a KD does not result in loss of DA neurons in zebrafish, although we did observe reorganization in the DA neuron distribution profile. The latter is most likely caused by spatial restrictions due to the brain ventricle dilation, also noted in the Tg(gfap:GFP) Atp1a3a- and Atp1a3b-KD embryos.

We also identified novel proteomic changes associated with the α3 isoform deficiencies. This is in fact the first time a proteomic approach is used to identify proteins up or down-regulated in ATP1α3-deficient cells. Interestingly, a recent study explored proteomic changes in ATP1α3-deficient zebrafish (18), and some of the regulated proteins, e.g. parvalbumin, muscle creatine kinase, published in that study, were also detected as regulated in Atp1a3b deficient embryos, indicating that the link between these proteins and the Na+/K+-ATPase is most likely dependent on the Na+/K+-ATPase pump function, rather than an isoform specific association. Hence, although preliminary, it provides an important initiative to be further assessed. To functionally address these candidate proteins in relation to the obtained Atp1a3a- and Atp1a3b-KD zebrafish and other animal models will be a future direction of this project.

Of particular interest is the down-regulation of parvalbumin detected in our proteomics assay of the Atp1a3a-KD embryos. Parvalbumin is expressed preferentially in a subpopulation of GA-Bergic neurons in mice, overlapping with the α3Na+/K+-ATPase expression (8,61,62). Parvalbumin modulates short-term synaptic plasticity (63), and thus compliments the signaling role of the α3Na+/K+-ATPase in synaptic plasticity and in dendritic growth in cortical neurons (64). Neuronal associations of α3Na+/K+-ATPase are further supported by the down-regulation of guanidinoacetate methyltransferase (GAMT). The guanidinoacetate (GAA) is the principal metabolite accumulating in guanidinoacetate methyltransferase (GAMT) deficiency, which significantly inhibits Na+/K+-ATPase activity (65). It was proposed that such inhibition may be one of the mechanisms involved in the neuronal dysfunction observed in patients suffering from GAMT deficiency, which shows symptoms comparable to RDP; e.g. muscle weakness, epilepsy and seizures (65,66).

Although it is clear that zebrafish embryos will not develop a full range of complex, human-like disorders, they can be used to study certain biological markers (endophenotypes) of these disorders. Indeed, several features of zebrafish α3 isoforms are comparable to the mammalian counterparts. This study comprehensively examined the spatial distribution of the zebrafish ATP1A3 orthologs and is the first report to show that the two distinctively expressed α3 isoforms caused enlarged brain ventricle when gene functions were diminished. This was accompanied with abrupt embryonic motility, most likely linked to depolarized RMP, as shown for the spinal RB neurons, and this, combined with our proteomic data, highly promote zebrafish as a relevant model to further assess α3Na+/K+-ATPase in brain development, neuronal excitability, and thus functions.
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FOOTNOTES
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FIGURE LEGENDS

FIGURE 1. Expression of Atp1a3a and Atp1a3b mRNA in zebrafish embryos. A. Atp1a3a (black bars) and Atp1a3b (grey bars) mRNA expressions were quantified by qRT-PCR and normalized to Actb2 expression. Data are presented as mean±s.e.m. of triplicate measurements. B. Atp1a3a mRNA expression analyzed by whole mount in situ hybridization in 60 hpf zebrafish embryos; insert shows sense probe hybridized control embryo. Atp1a3a is expressed in the brain and the spinal cord. The numbered vertical dashed lines, here and in C, show the positions of the transverse sections shown below in I-V. C. Atp1a3b mRNA expression analyzed by whole mount in situ hybridization in 60 hpf zebrafish embryos; insert shows sense probe hybridized control embryo. Atp1a3b is expressed in specific brain regions. Scale bars represent 100 µm in whole-mount images and 50 µm in sections.

FIGURE 2. Knockdown of Atp1a3a causes brain ventricle dilation. A. Significant brain ventricle dilation occurred in embryos upon Atp1a3a KD mediated by α3a-MO or α3a-SP-MO, compared to std-MO injected control embryo. This phenotype was rescued by co-injection of Atp1a3a mRNA. p53-MO co-injections with any of the MOs did not rescue the brain ventricle dilation phenotype. B. Brain ventricles of Tg(gfap:GFP) line, non-MO injected and α3a-MO injected, were injected with rhodamine-conjugated dextran. Brain ventricles and the astrocytes are highlighted by red and green fluorescence respectively. C. qRT-PCR tested efficiency of α3a-SP-MO mediated KD in terms of changes in the relative expression level of Atp1a3a. Data are presented as mean±s.e.m. of triplicate measurements. Concentrations of MOs are indicated. D. Mean percentages±s.d. of the α3a-SP-MO injected embryos suffering from brain ventricle dilation of different severity, with (white columns, n=117) and without (black columns, n=96) Atp1a3a mRNA co-injection, are plotted. Embryos at the lower panel represent the extend of the brain ventricle dilation used as a criteria for grouping as severe (++) (scale bar: ~235 µm), moderate (++) (scale bar: ~150 µm) and slight/no (+) (scale bar: ~65 µm).

FIGURE 3. Knockdown of Atp1a3b phenocopies Atp1a3a knockdown. A. Brain ventricle dilation was observed in embryos upon Atp1a3b KD mediated by α3b-MO or α3b-SP-MO, compared to the std-MO injected control embryo. This phenotype was rescued by co-injection of Atp1a3b mRNA. p53-MO co-injections with any of the MOs did not rescue the brain ventricle dilation phenotype. B. Brain ventricles of Tg(gfap:GFP) line, non-MO injected and α3b-MO injected, were injected with rhodamine-conjugated dextran. Brain ventricles and the astrocytes are highlighted by red and green fluorescence respectively. C. α3b-SP-MO mediated KD efficiency was tested by qRT-PCR in terms of changes in the relative expression level of Atp1a3b. Data are presented as mean±s.e.m. of triplicate measurements. Concentrations of MOs are indicated. D. Mean percentages±s.d. of the α3b-SP-MO injected embryos suffering from brain ventricle dilation of different severity, with (white columns, n=148) and without (black columns, n=88) Atp1a3a mRNA co-injection, are plotted. The degree of brain ventricle dilation: +, slight/no; ++, moderate; ++++, severe. E. Atp1a3a mRNA did not rescue α3b-SP-MO injected embryos, similarly Atp1a3b mRNA did not rescue α3a-SP-MO injected embryos from brain ventricle dilation.

FIGURE 4. RB neurons are more depolarized in Atp1a3a-KD zebrafish displaying severe brain ventricle dilation. A. RMP values of RB neurons from WT (n=5), std-MO injected (n=6) and α3a-MO injected...
embryos (n=10) are plotted. The α₃a-MO injected embryos are divided into embryos displaying severe (+++) (n=5) or slight/no (+) (n=5) brain ventricle dilation. The number of cells (n) recorded per group stems from at least three different animals. RMP data are presented as mean±s.d. ** p<0.01 between RMPs of α₃a-MO injected embryos with severe ventricle dilation and control groups, WT and std-MO injected embryos. B. Schematic representation summarizing the depolarization of the RMP in RB neurons in an Atp1a3a-KD embryo. The scheme covers the time frame of 48-60 hpf. Dashed cross marks a malfunctioning α₃aNa⁺/K⁺-ATPase.

FIGURE 5. Atp1a3a- and Atp1a3b-KD embryos respond to touch but have abnormal motility. Successive frame shots from touch response assay display three representative WT embryos with burst swimming response (top panel, Movie S1), a representative α₃a-MO injected embryo that kept swirling around itself (middle panel, Movie S3), and a α₃b-MO injected embryo that responded as a short distance recoil (bottom panel, Movie S4). Touch-stimulated embryos are marked with a color coded asterisk and the frame shot times are merged on images in seconds.

FIGURE 6. Atp1a3a mRNA is present in DA neurons, but no loss of DA neuron was observed in Atp1a3a-KD embryos. A. TH-positive DA neurons (green fluorescence), and Atp1a3a mRNA expression (red fluorescence) in the zebrafish brain are imaged along the dorsoventral axis (anterior to the left; posterior to the right). Scale bars represent 50 μm. Areas marked by squares are shown in the lower panel at higher magnification (scale bars represent 5 μm). B. Th mRNA expression analyzed by in situ hybridization in WT, α₃a-SP-MO injected, and Atp1a3a mRNA-rescued embryos. C. Total staining of Th mRNA in WT (n=10) and α₃a-SP-MO injected embryos (n=10) was quantified using ImageJ and mean values of integrated densities are plotted with standard deviations.

FIGURE 7. Relative mRNA expressions of some of the regulated proteins detected in proteomics assay. mRNA expressions of selected proteins regulated by A. α₃a-MO mediated Atp1a3a KD or B. α₃b-MO mediated Atp1a3b KD were quantified by qRT-PCR and normalized to Actb2 expression in embryos at 60 hpf. Data are presented as mean±s.e.m. of triplicate measurements.
Table 1. List of regulated proteins selected from proteomics assay. Proteins relevant for the Atp1a3a- and Atp1a3b-KD phenotypes were selected from Table S1. Proteins were detected by iTRAQ LC-MS/MS. Database accession numbers, gene identity, relative fold change and tissue associations are given. The Atp1a3-KD/control protein expression ratio indicates up-regulation when it is equal/above 2 and down-regulation when it is equal/below 0.5.

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Figure 1

Zebrafish Na pump maintains brain ventricle volume and motility
Zebrafish Na pump maintains brain ventricle volume and motility

Figure 2

A

B

C

D

\( \alpha_3a \)-MO mediated knockdown

\( \alpha_3a \)-SP-MO mediated knockdown

Atp1a3a mRNA rescue

Knockdown

mRNA rescue

WT

\( \alpha_3a \)-MO 5.5 ng

\( \alpha_3a \)-MO 11 ng

\( \alpha_3a \)-SP-MO 5.5 ng

\( \alpha_3a \)-SP-MO 11 ng

\( \alpha_3a \)-MO+p53-MO

\( \alpha_3a \)-SP-MO+p53-MO
**Figure 3**

A. Comparison of std-MO and Atp1a3b mRNA rescue.

B. Expression analysis of Atp1a3b in different conditions.

C. Graph showing 3b-SP-MO mediated knockdown.

D. Graph showing Atp1a3b mRNA rescue.

E. Comparison of 3a-SP-MO+Atplab mRNA and 3b-SP-MO+Atplab mRNA.
**Zebrasne Na pump maintains brain ventricle volume and motility**

**Figure 4**

A. Graph showing values of Resting membrane potential (RMP) for different conditions:

- WT: -47 mV
- std-MO: -72.1 mV
- a3a-MO (+ VD): -46.6 mV
- a3a-MO (+++ VD): -46.6 mV

B. Diagram illustrating the regulation of RMP and neuronal excitability:

- Depolarized RMP
- Altered neuronal excitability

- [Na+]in, [K+]in

- RB neurons

- Atp1a3a-KD
Zebrafish Na pump maintains brain ventricle volume and motility
Figure 6

Zebrafish Na pump maintains brain ventricle volume and motility
Figure 7

Zebrafish Na pump maintains brain ventricle volume and motility

A B

0.0 0.2 0.4 0.6 0.8 1.0 1.2 1.4 1.6 1.8 2.0 2.2 2.4 2.6

Krd Krd5 1/3d7 A Bhtm Chima Chimb Gemt Gpi0 Mhiz2 Nopp2 Pgam2 Pve10 G9i3000 1/3d7 A

α3a-MO/α3b-MO

Std-MO
α3Na+/K+-ATPase deficiency causes brain ventricle dilation and abrupt embryonic motility in zebrafish
Canan Doganli, Hans C. Beck, Angeles B. Ribera, Claus Oxvig and Karin Lykke-Hartmann

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