Vesicular monoamine transporter 2 (SLC18A2) regulates monoamine turnover and brain development in zebrafish

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Abstract

Aim: We aimed at identifying potential roles of vesicular monoamine transporter 2, also known as Solute Carrier protein 18 A2 (SLC18A2) (hereafter, Vmat2), in brain monoamine regulation, their turnover, behaviour and brain development using a novel zebrafish model.

Methods: A zebrafish strain lacking functional Vmat2 was generated with the CRISPR/Cas9 system. Larval behaviour and heart rate were monitored. Monoamines and their metabolites were analysed with high-pressure liquid chromatography. Amine synthesising and degrading enzymes, and genes essential for brain development, were analysed with quantitative PCR, in situ hybridisation and immunocytochemistry.

Results: The 5-bp deletion in exon 3 caused an early frameshift and was lethal within 2 weeks post-fertilisation. Homozygous mutants (hereafter, mutants) displayed normal low locomotor activity during night-time but aberrant response to illumination changes. In mutants dopamine, noradrenaline, 5-hydroxytryptamine and histamine levels were reduced, whereas levels of dopamine and 5-hydroxytryptamine metabolites were increased, implying elevated monoamine turnover. Consistently, there were fewer histamine, 5-hydroxytryptamine and dopamine immunoreactive cells. Cellular dopamine immunostaining, in wild-type larvae more prominent in tyrosine hydroxylase 1 (Th1)-expressing than in Th2-expressing neurons, was absent in mutants. Despite reduced dopamine levels, mutants presented upregulated dopamine-synthesising enzymes. Further, in mutants the number of histidine decarboxylase-expressing neurons was increased, notch1a and pax2a were downregulated in brain proliferative zones.

Conclusion: Lack of Vmat2 increases monoamine turnover and upregulates genes encoding amine-synthesising enzymes, including histidine decarboxylase. Notch1a and pax2a, genes implicated in stem cell development, are downregulated in mutants. The zebrafish vmat2 mutant strain may be a useful model to study how monoamine transport affects brain development and function, and for use in drug screening.

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1  |  INTRODUCTION

The vesicular monoamine transporter 2 (VMAT2), renamed as solute carrier protein 18 A2 (SLC18A2) is a transmembrane protein and key regulator of the monoaminergic systems. It is mainly expressed in the CNS, uses an electrochemical gradient to transport cytoplasmic monoamines into presynaptic vesicles, and has been linked to the pathophysiology of different brain disorders. Reports of impaired VMAT2-mediated packaging of dopamine into vesicles and increased cytosolic dopamine turnover in Parkinson’s disease (PD) patients, and the neuroprotective effect generated by increased VMAT2 level or function, support a role for this protein in the pathophysiology of PD. Consistent with this possibility, a single nucleotide polymorphism in the promoter of VMAT2 was found to be associated with PD risk in Caucasians. VMAT2 blockers reserpine and tetrabenazine induce depressive symptoms in both humans and animals, and altered pharmacodynamics of VMAT2 binding in platelets of children with depression have been reported. Additionally, polygenic variants of the VMAT2 locus are associated with alterations of drinking behaviour and development of opioid dependence, implying a role for VMAT2 in drug addiction. Finally, VMAT2 is expressed in insulin-secreting beta cells of the pancreas and may safeguard them against dopamine toxicity during stress induced by a high-fat diet.

To investigate the consequence of abnormal VMAT2 on embryonic development and adult physiology several lines of Vmat2 mutant mice have been generated. Mice homozygous for loss-of-function alleles of Vmat2 do not grow into adulthood. In the neonatal brain, mutants relative to wild types display a 94%-99% reduction of dopamine (DA), noradrenaline (NE) and serotonin (5-HT) levels. Additionally, mutants are hypoactive and unable to feed properly. In heterozygous Vmat2 mutants, a less drastic reduction in DA (42%), NE (23%) and 5-HT (34%) levels has been reported. Heterozygous mice are viable and grow into adulthood, and as adults display a depressive-like phenotype and increased sensitivity to parkinsonian toxins.

CRISPR/Cas9 technology has been recently applied to modify the vmat2 gene in zebrafish. The zebrafish (Danio rerio) has become a popular model organism in neuroscience because it shares genetic homology with humans and possesses the main neurotransmitters present in humans. Its transparency during embryonic stages and rapid external development allow in vivo visualisation of cell biological events. Additionally, genetic tools are available to establish mutant lines in a relatively short time and they are amenable to relatively high-throughput drug screening. Wang et al analysed biochemical and behavioural parameters of adult heterozygous animals, which presented with an anxiety-like phenotype and a significant reduction in DA, 5-HT and NE levels. Analyses of homozygous vmat2 mutant zebrafish have not been previously reported. Here we report creating a loss of function allele of vmat2/slc18a2 and evaluate the dopaminergic, serotonergic and histaminergic systems, as well as behavioural parameters, in larvae homozygous for this allele.

2  |  RESULTS

2.1  |  Vmat2 mutants

We used the CRISPR/Cas9 system in outbred zebrafish to generate a 5-base-pair (5-bp), frameshift-inducing deletion in exon 3 of slc18a2 (Figure 1B). The longest open reading frame in the zebrafish slc18a2 mRNA (Genbank Accession BC090766) encodes a polypeptide of 562 amino acids (Genbank Accession number AHH90766). However, the first methionine in this predicted polypeptide begins a frameshift near the initial 5- base-pair (5-bp) region. In the homozygous zebrafish have 515 amino acids and one aberrant one. All progenies were tail-clipped at 3 dpf and genotyped using HRM analysis, resulting in distinct melting curves of each genotype (Figure 1A). Genotypes were also confirmed by the absence of restriction digestion in the mutant individuals (Figure 1C). In 6 days post-fertilisation (dpf) larvae homozygous for this mutation (hereafter, vmat2+/- larvae or vmat2 mutants), vmat2 mRNA levels were significantly reduced relative to vmat2+/+ siblings (n = 5 for each genotype; Figure 1D), presumably because of nonsense-mediated decay. This result was supported by a clearly weaker in situ hybridisation mRNA signal in the brains of 6 dpf vmat2+/- larvae compared with vmat2+/+ siblings (n = 6 for each genotype; Figure 1B,C). The homozygous vmat2 mutation was lethal between the larval and juvenile stages of development. Video analysis of anesthetised larvae (Video S1) revealed an increased heart rate in vmat2+/- relative to in vmat2+/+ siblings (Video S2) (n = 7 for each genotype; Figure 1E).
(A) Normalized and Temp-Shifted Difference Plot

(B) Vmat2+/+

(C) DNA marker

(D) Vmat2

(E) Vmat2+/+

(F) Vmat2−/−

(G) Heart rate
2.2 | Behavioural analysis

We measured overall activity over 24 hpf. In both day and night, vmat2\(^{-/-}\) larvae were hypoactive relative to vmat2\(^{+/+}\) sibling larvae during the daytime (n = 16 for each genotype; Figure 2A). We next evaluated behaviour in a dark-flash paradigm. After a sudden switch from bright light to darkness, 5 dpf and older larvae respond with a startle motion. Here we tested 6 dpf larvae with several cycles of lights being switched on and off between 12:00 and 16:00. Mutants displayed a greater increase in locomotion during after the switch to darkness relative to vmat2\(^{+/+}\) siblings (n = 16 for each genotype; Figure 2B); this pattern was repeated over several cycles (Figure 2B).

2.3 | Histaminergic system

We next examined the expression of monoamines and relevant enzymes, beginning with the histamine system. In the larval brains at 6 dpf, vmat2\(^{-/-}\) fish relative to vmat2\(^{+/+}\) siblings displayed a reduction in the number of histamine

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**FIGURE 1** Vmat2 mutants. A, Results of the HRM analysis showing the distinctive melting curves of each genotype. B, Sequencing chromatograms of tail-clipped DNA showing the sequence and the newly generated stop codon of a vmat2\(^{-/-}\) zebrafish. C, Identification of genotypes by restriction digestion of PCR products. D, Bar charts showing results of reverse transcription-quantitative PCR (RT-qPCR) analysis on larvae of the indicated genotype at 6 dpf. Vmat2\(^{-/-}\) larvae exhibit 60% less vmat2 transcript than vmat2\(^{+/+}\) larvae. E and F, Ventral views of whole-mount 6 dpf larval brains, anterior to the left, processed for vmat2 RNA in situ hybridisation (ISH). Vmat2 expression was clearly lower in mutants than in vmat2\(^{+/+}\) larvae. Scale bar = 75 µm. n = 5 for each genotype. G, Bar chart showing heart rate in larvae of the indicated genotype at 6 dpf. n = 7 for each genotype. The heart rate was lower in mutants than in vmat2\(^{+/+}\) siblings. Data are mean ± SEM. Student’s t test was used for statistical analysis. \(^1P < .01\). \(^2P < .001\)
(A) (B) | (C) (D) | (E) (F) | (G) | (H) | (I) | (J)
immunoreactive cell bodies (n = 10 for each genotype, Figure 3G), and fibres in the ascending, descending, and commissural fibre networks, and in the major terminal network in the telencephalon (Figure 3A,B). Histamine levels detected by HPLC were also strongly reduced in mutants compared to vmat2+/+ siblings (n = 6 for each genotype) (Figure 3I). Interestingly, despite the reduction of histamine, the number of cells expressing hdc mRNA (encoding Histidine decarboxylase, the enzyme responsible for catalysing the conversion of histidine to histamine) was higher in the brains of the vmat2+/− larvae than in vmat2+/+ siblings (n = 6 for each genotype; Figure 3C,D), implying the existence of a feedback circuit meant to maintain histamine levels. By contrast, RT-qPCR analysis indicated the levels of hdc-positive cells counting on larval brains of the indicated genotype. The number of hdc-positive cells was significantly increased in the vmat2+/− brains. n = 6 for each genotype. I, Bar chart showing results of high-performance liquid chromatography (HPLC) assay on larvae of the indicated genotype at 6 dpf. The number of histaminergic neurons was significantly reduced in the brains of vmat2+/− larvae at 6 dpf. Histamine level was significantly decreased in vmat2+/− larvae. n= 6 for each genotype. J, Bar chart showing results of reverse transcription-quantitative PCR (RT-qPCR) assays on larvae of the indicated genotype at 6 dpf. Vmat2+/− and vmat2+/+ did not show a significant difference in the amount of hrh1 transcript. Data are mean ± SEM. Student's t test was used for statistical analysis. *P < .05. †P < .001

Tyrosine hydroxylase catalyses the first and rate-limiting step in the synthesis of dopamine and other catecholamines; zebrafish have two paralogs, Th1 and Th2. RT-qPCR on lysates of whole larvae indicated an upregulation of th1 and th2 mRNA in the vmat2+/− group (n = 5 for each genotype; Figure 4M,P). However, the immunostaining for Th1 (n = 7 for each genotype; Figure 4H-J) and RNA in situ hybridisation patterns for th1 (n = 5 for each genotype; Figure 4K,L) were not grossly different between vmat2+/+ and vmat2+/− groups. By contrast, RNA in situ hybridisation showed a stronger th2 mRNA signal in the brains of vmat2+/− compared to in vmat2+/+ larvae, particularly in the neuron groups 9b and 10b, in the posterior hypothalamus (n = 5 for each genotype; Figure 4N,O).

RT-qPCR and in situ hybridisation showed that there were no differences in dopamine transporter (dat) between genotypes at 6 dpf (Figure S2). Similarly, no differences between the groups were detected in levels of adrenaline (ADR), homovanillic acid (HVA) and 3-Methoxytyramine (3MT) (n = 6 for each genotype, data not shown).

To reveal whether Th1 or Th2 is responsible for the production of dopamine in the most prominent dopamine-containing neuron groups, we first assessed the relative distributions of dopamine and Th1 immunoreactivity in doubly-stained 6 dpf WT Turku zebrafish larvae (n = 6 for each genotype; Figure 5). The dopamine-containing neurons were most numerous in the posterior hypothalamus (group 10b), which contained very few Th1 immunoreactive neurons but many that express th2, suggesting that tyrosine hydroxylase 2 (Th2) is responsible for the production of dopamine in a majority of these neurons. In the more rostral cell groups (group 8), Th1 immunoreactive neurons were found, many immunoreactive for dopamine, suggesting Th1 mediates dopamine synthesis in these cells (Figure 5). All dopamine-producing cell groups, both those expressing th1 and those expressing th2, lost almost all anti-dopamine immunoreactivity in mutant larvae (Figure 4A,B).

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2.5 | Serotonergic system

Concomitantly with the decline in the number of histamine and dopamine immunoreactive cells, the number of 5-HT immunoreactive cells was strongly reduced in the \( \text{vmat2}^{-/-} \) group relative to \( \text{vmat2}^{+/+} \) siblings, to the extent that they were essentially absent from mutants (\( n = 6 \) for each genotype; Figure 6A,B). Consistent with a compensatory upregulation of Th expression in \( \text{vmat2} \) mutants, expression of \( \text{tph1a} \), encoding tryptophan hydroxylase, the rate-limiting enzyme in serotonin synthesis, was clearly stronger signal in \( \text{vmat2}^{-/-} \) larvae than in their \( \text{vmat2}^{+/+} \) siblings (\( n = 5 \) for each genotype; Figure 6C,D), particularly on group 3.\(^{22}\) 5-HT was significantly decreased and metabolite 5-HIAA level was higher in the \( \text{vmat2}^{-/-} \) group than in \( \text{vmat2}^{+/+} \) siblings (\( n = 6 \) for each genotype; Figure 6E,F).
FIGURE 4 The Catecholaminergic system. A and B, Ventral views of whole-mount 6 dpf larval brains, anterior to the left, processed for dopamine (DA) immunostaining. Dopamine-immunoreactive neurons are present in the diencephalon and telencephalon with extensive descending and ascending fibre networks in the vmat2+/+ larval brain, but only scattered dopamine-immunoreactive neuronal cell bodies are visible in the vmat2−/− larval brain. Scale bar = 75 µm. C-E, Bar chart showing results of high-performance liquid chromatography (HPLC) assay on larvae of the indicated genotype at 6 dpf. Levels of dopamine (DA), noradrenaline (NA) and DOPAC. n = 6 for each genotype. F and G, Bar charts showing results of reverse transcription-quantitative PCR (RT-qPCR) assays on larvae of the indicated genotype at 6 dpf. Vmat2+/+ and vmat2−/− larvae presented similar levels of conta and mao transcripts. n= 5 for each genotype. H and I, Ventral views of whole-mount 6 dpf larval brains, anterior to the left, processed for tyrosine hydroxylase (Th1) immunostaining. Scale bar = 75 µm. J, Bar chart showing results of Th1-immunoreactive cells counting on larval brains of the indicated genotype at 6 dpf. n = 7 for each genotype. K and L, Ventral views of whole-mount 6 dpf larval brains, anterior to the left, processed for tyrosine hydroxylase (th1) RNA in situ hybridisation (ISH). Diencephalic neuronal clusters express th1 in vmat2+/+ and vmat2−/− larval brains. Scale bar = 75 µm. M, Bar charts showing results of reverse transcription-quantitative PCR (RT-qPCR) assay on larvae of the indicated genotype at 6 dpf. Vmat2−/− larvae exhibit increased levels of th1 transcript when compared to their vmat2+/+ siblings. n= 5 for each genotype. N and O, Ventral views of whole-mount 6 dpf larval brains, anterior to the left, processed for tyrosine hydroxylase 2 (th2) RNA in situ hybridisation (ISH). Scale bar = 75 µm. P, Bar charts showing results of reverse transcription-quantitative PCR (RT-qPCR) assay on larvae of the indicated genotype at 6 dpf. Vmat2+/+ larvae exhibit increased levels of th2 transcript when compared to their vmat2−/− siblings. n= 5 for each genotype. Data are mean ± SEM. Student’s t test was used for statistical analysis. *P < .05. †P < .01. ††P < .001

FIGURE 5 Dopamine and th1 immunoreactivity in the hypothalamus of 6 dpf WT Turku zebrafish larvae. A-C, Ventral views of whole-mount 6 dpf larval brains, anterior to the left, processed for dopamine (DA) and tyrosine hydroxylase (Th1) double immunostaining. Sample fixed with a combination of glutaraldehyde and paraformaldehyde to allow visualisation of both dopamine and th1. It is noteworthy that the fixation required for double staining compromised the signal from both antibodies compared with conditions under which both antibodies perform optimally. Most dopamine-containing neurons are seen in the posterior hypothalamic group 10b, which contains very few weakly Th1-immunoreactive neurons. In more rostral neuron groups (arrowheads) both Th1 and dopamine are seen, and some neurons clearly contain both markers (small arrows). Scale bar = 75 µm

2.6 Developmental markers

Next, based on evidence that VMAT2 inhibits differentiation of pancreatic precursor cells and on the extensive literature indicating that monoamines modulate neuronal differentiation and proliferation, we aimed to verify the effects of vmat2 deletion on brain development. Notch1 signalling contributes to the maintenance of neuronal precursor cells (NPCs) in an undifferentiated state by inhibiting neuronal differentiation, while its suppression stimulates differentiation of neuronal precursors. Interestingly, expression of early neuronal marker notch1a was clearly weaker in the brains of vmat2−/− 6 dpf larvae when compared with their vmat2+/+ siblings (n = 5 for each genotype; Figure 7C,D). RT-qPCR analysis confirmed that notch1a expression was significantly downregulated in vmat2−/− larvae (n = 5 for each genotype; Figure 7G). Reduced notch1a levels in vmat2 mutants implies that Vmat2-dependent monoaminergic signalling normally suppresses the differentiation of these neurons.

Moreover, expression of pax2a, encoding a key transcription factor for the development of midbrain dopaminergic neurons, was also apparently weaker in the brains of vmat2−/− larvae in comparison with their vmat2+/+ siblings (n = 5 for each genotype; Figure 7E,F). RT-qPCR analysis revealed that mutants expressed higher levels of mesencephalic astrocyte-derived neurotrophic factor (manf), encoding a protein that promotes differentiation of several types of neurons including dopaminergic ones (n = 5 for each genotype; Figure 7H). No differences between genotypes were detected when we measured the levels of the proliferation marker proliferating cell nuclear antigen (pcommercially contacting) and glial fibrillary acidic protein (gfap) (n = 5 for each genotype; Figure 7I,J). The panneuronal marker HuC was also analysed by immunostaining, but no evident alteration on its pattern was detected in the brains of vmat2−/− larvae when compared to their vmat2+/+ siblings (n = 5 for each genotype; Figure 7A,B).
In this study, a \textit{vmat2} knockout zebrafish was generated using the CRISPR/Cas9 gene-editing method and the morphological, behavioural and neurological characterisation of these animals at the early developmental stage was reported. The \textit{vmat2} deficiency caused severe impairments in larval monoaminergic systems and behaviour and was lethal within 2 weeks post-fertilisation. Although our findings on the aminergic systems are reminiscent of the phenotype reported in well-established rodent models that lack \textit{vmat2},
\cite{10,11,13} several new findings were made. These include upregulation of \textit{hdc} expression and decreased expression of \textit{notch1a}, encoding a protein that regulates stem cell populations.

Zebrafish larvae have the innate tendency to increase their locomotion in response to a sudden change in the illumination of their environment. During a period of sudden darkness a burst of locomotor activity, which is referred to as “dark-flash” response, is normally observed.\cite{18} While ablation-studies indicate this behaviour is not dependent on the Mauthner cells, which are known to modulate rapid escape reactions in the zebrafish,\cite{18} previous pharmacological evidence suggest that disrupted monoaminergic signalling leads to a stronger dark-flash response.\cite{32,33} In our genetic study, the \textit{vmat2}−/− larvae displayed a stronger dark-flash response than their \textit{vmat2}+/+ siblings, reinforcing the findings from pharmacological studies.

VMAT2 plays a crucial role in monoaminergic signalling. Most studies focus on dopamine, 5-HT and/or noradrenaline. However, two studies of the histaminergic system, using different models but both using reserpine to inhibit VMAT2 activity, reported either decreased\cite{34} and or unchanged\cite{35} levels of histamine after drug treatment. In the current study, we used a zebrafish \textit{vmat2} mutant to evaluate the status of the histaminergic system in the absence of this monoaminergic transporter. Histamine immunoreactivity in the brain was strongly reduced in \textit{vmat2} mutants both in neuronal cell bodies and their main projections to the dorsal telencephalon and habenula. Interestingly, the immunoreactivity in cell bodies was not completely absent, with some cells presenting a clear cytoplasmic immunoreactivity. The presence of immunoreactivity in some neurons could indicate the existence of a still uncharacterised mechanism of transport-specific for histamine present in a subset of histaminergic neurons. The present results are in agreement with the previous data on WT zebrafish treated with reserpine during an early developmental stage.\cite{33} The HPLC data showed an 85% reduction in the amount of histamine in \textit{vmat2}−/− larvae when compared with \textit{vmat2}+/+ siblings. Concomitantly, the number of \textit{hdc}-positive cells in the brains of \textit{vmat2} mutants was increased by 30%, perhaps reflecting a homeostatic mechanism to upregulate histamine signalling. This phenomenon was not seen in reserpine-treated larvae.\cite{33} It is noteworthy that \textit{hdc} mRNA expression in a cell does not directly indicate the rate of histamine production, which is also dependent on post-translational processing of Hdc and substrate availability. Interestingly, the drastic decline in histamine and a subsequent impaired histaminergic transmission in \textit{vmat2} mutants did not have an apparent effect on the expression of \textit{hrh1}, which encodes a histamine receptor.

In zebrafish, the \textit{tyrosine hydroxylase} (\textit{th}) gene is duplicated and these animals possess Th1 and Th2 enzymes. Expression of distinct paralogs predominates in different brain regions. For instance, in the hypothalamus of adult zebrafish, most dopamine-immunoreactive cells express Th2,\cite{36} and \textit{th2} knockdown significantly reduces the number of dopamine-immunoreactive cells in the hypothalamus of zebrafish larvae.\cite{37} Consistent with these findings, here we showed that in the hypothalamus of WT Turku zebrafish...
larvae at 6 dpf only a minority of dopamine immunoreactive cells were also immunoreactive for Th1. Together these results show that Th2 is the paralog primarily responsible for dopamine synthesis in the hypothalamus.

Both dopamine levels and immunostaining showed drastic decreases in the brains of the vmat2−/− larvae. However, when considering dopamine metabolites, DOPAC was significantly increased, indicating an accelerated dopamine turnover. In addition, RT-qPCR showed upregulated th1 and th2 mRNA levels in vmat2−/− samples relative to vmat2+/+ samples. A similar phenomenon has been described in PD patients, with upregulation of TH mRNA positive neurons in the ventral tegmental area. Enhanced TH activity has been reported in VMAT2 KO mice, which presented an increased accumulation of levodopa. Of note, Th1 expression is not limited to the brain, and therefore an increase of th1 levels in lysates of whole embryos could reflect an upregulation of this gene in organs other than the brain.

We also found that there was no cellular 5-HT immunoreactivity in the brains of vmat2−/− fish and that 5-HT levels were drastically decreased in mutants relative to their vmat2+/+ siblings. The HPLC results also showed that the metabolite 5-HIAA was significantly increased. Serotonergic dysfunction is commonly reported in patients with PD. The degeneration of serotonergic terminals starts at an early stage of the disease course and is associated with depressive symptomatology, weight and appetite problems, fatigue, and visual hallucinations displayed by the patients. In situ hybridisation showed a clearly stronger mRNA signal for tph1a in the brains of vmat2−/− larvae, similarly with hdc, th1 and th2 upregulations. Vmat2 inhibition through reserpine treatment leads to increased TH mRNA expression in the substantia nigra and locus coeruleus and to enhanced TH activity. The increase in the activity of these enzymes is thought to result from the lack of monoamine release, which is known to inhibit presynaptic autoreceptors involved in the regulation of enzymatic activity. The impairment of different monoaminergic systems might have led to an intrinsic compensatory mechanism aiming at self-repair, since we detected upregulation of genes related to synthesis enzymes of monoamines. The mRNA levels of mao and comt were not significantly different when vmat2+/+ and vmat2−/− larvae were compared. The monoaminergic depletion detected in vmat2−/− larvae is likely to have affected peripheral organs. In zebrafish, vmat2 is present in arch-associated neurons rostral to the heart. Anesthetised vmat2−/− larvae displayed a greater heart rate when compared with vmat2+/+ siblings. Similarly,
mouse Vmat2 mutants presented with increased heart rates, systolic, diastolic and mean femoral arterial blood pressures when compared to WT animals.\textsuperscript{44} It has been proposed that allelic variants of the VMAT2 gene might candidates for studies seeking gene differences that could predispose to human long QT syndromes, cardiac arrhythmias, and sudden death.\textsuperscript{45} Zebrafish vmat2 mutants could be a useful tool in future studies aiming to understand the role of this transporter in cardiac function, because of the similarities in heart rate and action potential duration and morphology with respect to humans.\textsuperscript{46} Furthermore, it is possible to perform ECGs on zebrafish embryos from microelectrode recordings as early as 3 dpf, in which there can be diffusional drug delivery.\textsuperscript{47}

One of the most intriguing results of this study is evidence that VMAT2 contributes to a pathway that maintains the quiescence of neuronal precursors. During development, increased NOTCH activity is associated with quiescence of neural progenitor cell populations whereas its inhibition induces neuronal differentiation.\textsuperscript{48} Similarly in pancreatic progenitor cells depleted of Notch signalling neurogenin-3 (NGN3) expression is enhanced leading to the initiation of the endocrine lineage.\textsuperscript{49} Consistent with VMAT2 promoting Notch expression, VMAT2 inhibitors reserpine and tetrabenazine stimulate the differentiation of Pdx1+ cells into Ngn3+ cells, which quickly turn into Ins+cells.\textsuperscript{50} Here we found that zebrafish vmat2 mutants exhibit lower than normal levels of notch1a expression. Additionally, pax2a expression, an early marker of midbrain dopaminergic neurons, showed a weaker in situ hybridisation signal in the brains of vmat2\textsuperscript{−/−} zebrafish relative to their vmat2\textsuperscript{+/+} siblings. Dopaminergic neurons are derived from proliferating progenitors that sequentially express Pax2 and Pax5 and, upon exiting the cell cycle, become positive for engrailed-1 (En1) and eventually TH.\textsuperscript{51} Vmat2\textsuperscript{−/−} larvae also expressed higher than normal levels of manf, encoding a protein that promotes differentiation of neural progenitor cells,\textsuperscript{52} regulates the dopaminergic neuron development,\textsuperscript{31} regulates differentiation of pancreatic cells\textsuperscript{50} and has neuroprotective properties.\textsuperscript{51} In summary, the phenotype of zebrafish vmat2 loss-of-function mutants implicates VMAT2 in the regulation of neuronal differentiation in the brain.

4 | CONCLUSIONS

This is the first characterisation of a vmat2\textsuperscript{−/−} zebrafish mutant. We provided molecular and behavioural evidence that supports the use of this tool in future studies aiming to investigate basic mechanisms involving this monoamine transporter, screen potential new drugs or model brain disorders where VMAT2 plays a critical role. Importantly, we brought more information regarding the importance of VMAT2 to the histaminergic system, which has received less attention in comparison to the other monoaminergic systems in studies involving this transporter. Furthermore, the lack of vmat2 altered the expression of genes related to development and neurogenesis, indicating that this transporter might have a significant role in these processes, perhaps through amines normally stored by this transporter.

5 | METHODS

5.1 | Experimental animals

All animal care and experimental procedures complied with the ethical guidelines of the European convention and were approved by the Office of the Regional Government of Southern Finland (ESAVI/6100/04.10.07/2015). The vmat2 mutant was generated at the University of Iowa in outbred zebrafish (adults acquired from Aquatica Tropicals, Plant City, Florida). Subsequent generations were crossed into the wild-type (WT) Turku strain, which has been maintained in the Panula laboratory for more than 20 years and utilised in several studies.\textsuperscript{19,32} This work is conforming with good publishing practice in physiology.\textsuperscript{52}

5.2 | Generation of vmat2 mutants

The Cas 9 encoding pT3TS-nCas9n\textsuperscript{53} was linearised with XbaI (New England Biolabs) and purified using QIAquick Gel Extraction Kit (Qiagen). The mRNA was synthesised using T3 mMESSAGE mMACHINE (Life Technologies). The product was purified using RNeasy Mini Kit (Qiagen). The mRNA was synthesised using T3 mMESSAGE mMACHINE (Life Technologies). The product was purified using RNeasy Mini Kit (Qiagen). The vector pDR274\textsuperscript{54} was used to create the guide RNA. It was digested with BsaI (NEB) and isolated with QIAquick Gel Extraction Kit (Qiagen). The guide sequence was found using the following online design tools: CRISPR/ guides selection at crispr.mit.edu (based on Ref.55) and ZiFiT (based on Ref.56). The targeted guide sequence for exon 3 of slc18a2-001 (ENSDART00000025466) (vmat2) was 5’-ggacgacgaggctgctcagatgg-3′. The oligonucleotides used were 1:5’-taggacgacgaggctgctcagatgg-3′ and 2:5’-aaactctgagcagcctcgtcgt-3′. They were annealed to each other by heating to 95 degrees and then cooling to room temperature over 30 minutes. The annealed oligos were ligated into the vector backbone with T4 DNA ligase (NEB). The plasmid was transformed into DH alpha max competent cells (Invitrogen), grown, and extracted with the Wizard Miniprep DNA Purification Kit (Promega). The plasmids were sequenced at University of Iowa Institute of Human Genetics using Sanger sequencing with the primer M13 forward 5’-tgtaaaacgacggccagt-3′. The plasmids were linearised with HindIII (NEB) and purified with QIAquick Gel Extraction Kit (Qiagen). The MaxiScript T7 synthesis kit (Invitrogen) was
used to synthesise non-capped RNA. The product was purified with RNeasy minikit (Qiagen). The RNA concentration was quantified spectrophotometrically (NanoDrop) before storage at −80 degrees. The guide RNA (final concentration 12 ng/μL) and Cas9 RNA (final concentration 300 ng/μL) were co-injected into one-cell stage zebrafish embryos.

5.3 Genotyping

Larvae were genotyped at 3 days post-fertilisation (dpf) when genomic DNA was lysed after individual tail clippings were incubated in 50 μL lysis buffer (10 mmol/L Tris-HCl pH8.3, 50 mmol/L KCl, 0.3% Tween-20 and 0.3% NP-40) at 98°C for 10 minutes, followed by incubation on ice for 2 minutes. 1 μL of Proteinase K (20 mg/mL) was added to remove protein, and the mixture was incubated at 55°C for at least 4 hours. To inactivate Proteinase K, samples were incubated at 98°C for 10 minutes and quenched on ice. To detect mutations, high-resolution melting (HRM) curve acquisition and analysis was performed. Primers flanking the mutation site were designed using Primer-BLAST. High-Resolution Melt Analysis (HRMA) was performed on a LightCycler® 480 instrument (Roche) using the following reaction mixtures: 1x LightCycler 480 HRMA master mix (Roche), 2 mmol/L MgCl2 and 0.15 μmol/L primer mixtures. The PCR cycling protocol was as follows: one cycle of 95°C for 10 minutes; 45 cycles of 95°C for 10 seconds, 60°C for 15 seconds, 72°C for 20 seconds and melting curve acquisition; one cycle of 95°C for 60 seconds, and 40°C for 60 seconds. PCR products were denatured at 95°C for 60 seconds, renatured at 40°C for 60 seconds, and melted at 60-95°C with 25 signal acquisitions per degree. Melting curves were generated over a 65-95°C range. Curves were analysed using the LightCycler® 480 gene-scanning software (version 1.5) according to the manufacturer's instructions (Roche Diagnostics Ltd., Switzerland). To identify deviations of the curves indicative of sequence mutations, a three-step analysis was performed using the Gene Scanning programme (Roche) as follows: (1) Normalising the raw melting-curve data by setting the initial fluorescence uniformly to a relative value of 100% and the final fluorescence to a relative value of 0%. (2) Determining the temperature threshold at which the entire double-stranded DNA was completely denatured. (3) Further analysing the differences in melting-curve shapes (threshold setup 0) in order to cluster the melting curves with similar shapes into the same groups. Those with analogous melting curves were characterised as the same genotype.

Genotyping was also done on lysed tail-clipped DNA by the standard polymerase chain reaction (PCR). PCR was done using the Phusion Hot Start II DNA Polymerase kit (Thermo Fisher), using the forward primer 5’-ttctctctagtgcctattatcccaag-3’ and the reverse primer 5’-aggtgcctcgatggagaaga-3’. The PCR protocol was conducted based on the manufacturer's instruction. The PCR fragments were purified using QIAquick PCR Purification Kit (QIAGEN) and digested by Ddel (Thermo Fisher). The undigested and digested samples were subjected to 2% agarose gel electrophoresis. The digested wild-type samples displayed two bands 163 and 50 bp. The digested heterozygous samples displayed three bands 203, 163 and 43 bp. The digested homozygous mutants displayed a single band 205 bp because the Ddel site on the PCR product was deleted from the homozygous mutant embryos.

After genotyping, larvae were raised until 6 dpf and used in the experiments. All the comparisons were made between vmat2+/− larvae and their vmat2+/+ siblings. We attempted to verify a reduction of Vmat2 protein levels in homozygous mutants using a published VMAT2-sensitive fluorescent substrate and by Western blot analysis. However, the substrate proved to be insensitive to zebrafish Vmat2 and the available anti-Vmat2 antibodies did not recognise the zebrafish protein. We note that the mutation causes a frameshift in exon 3 at the codon encoding amino acid 62, of a protein anticipated to contain at least 562 amino acids (NCBI Accession: AAH90766), and induces non-sense mediated decay. Therefore, any protein produced in the mutants is expected to be at low levels and non-functional. The mutation was identified after the target locus was PCR-amplified from individual genomic DNA. PCR products were then cloned and sequenced.

5.4 Heart rate measurement

At 6 dpf, zebrafish larvae were anaesthetised in petri dishes with 0.02% tricaine. Larvae were transferred to glass slides, ensuring the presence of at least 100μL of liquid. The counting was done while observing the larvae under 10× objective of the Leica inverted microscope DMI1. Each heart rate measurement was done by counting the contractions of either of the two chambers for 20 seconds. Videos were recorded using a Leica digital HDMI camera attached to the back of the microscope.

5.5 Behavioural assays

The dark-flash response of larvae was evaluated at 6 dpf as described before. Briefly, after initial 5 minutes of basic locomotor activity tracking, larvae were exposed to alternating 2 minutes periods of darkness and light, and with three periods of darkness in total. The locomotor activity was analysed in 30 seconds bins. Larvae were individually tracked in 48-well plates using the DanioVision system and EthoVision XT software (Noldus Information Technology, Wageningen, the
Netherlands). This behavioural test was done between 12:00 and 16:00 with three independent biological replicates.

In the sleep evaluation, larvae of each genotype were tracked simultaneously for 24 hours, with the light conditions following the regular light/dark cycle of the larvae. The trial was started at 12:00 noon. The day and night activity was analysed in 60 minutes bins by calculating the total distance moved. Larvae were individually tracked using the DanioVision system and EthoVision XT software. This assay was repeated with an independent biological replicate.

### 5.6 Reverse transcription - quantitative PCR (RT-qPCR)

Larval zebrafish were killed at 6 dpf in ice-cold water prior to RNA extraction, and total RNA was extracted using the RNeasy mini Kit (Qiagen, Hilden, Germany). Larval samples (10 pooled 6 dpf larvae per sample) were collected and 1.0 μg of total RNA was reverse-transcribed using SuperScript™ III reverse transcriptase (Invitrogen, Carlsbad, USA). RT-qPCR was done with the LightCycler 480 real-time PCR system and the LightCycler 480 SYBR Green I master kit (Roche Applied Science, Mannheim, Germany). Primers for amplification were designed with Primer-BLAST (NCBI), and sequences are shown in Table 1. Cycling parameters were as follows: 95°C for 30 seconds and 45 cycles of the following, 95°C for 10 seconds and 62°C for 45 seconds. Fluorescence changes were monitored with SYBR Green after every cycle. Dissociation curve analysis was performed (0.2°C per s increase from 60 to 95°C with continuous fluorescence readings) at the end of cycles to ensure that only a single amplicon was obtained. All reactions were performed in duplicate. Results were evaluated with the LightCycler 480 Software version 1.5. Quantification was done by Ct value comparison, using the Ct value of ribosomal protein large subunit 13a (*rpl13a*) as the reference control.

### 5.7 Catecholamine and histamine measurement by high-performance liquid chromatography (HPLC)

Groups of 10 whole 6 dpf larvae were killed in ice-cold water and homogenised by sonication in 2% perchloric acid for each sample, centrifuged for 30 min at 15 000 g at 4°C, and filtered through a 0.45-μm PVDF filter (Pall Life Sciences, Ann Arbor, USA) before loading onto the HPLC system. All analyses were done in duplicate to ensure the reliability of values. The detection details have been described earlier. In order to normalise the results from HPLC measurement, we utilised the bicinchoninic acid assay kit (Thermo Fisher Scientific, Waltham, USA) to measure protein concentration.

### 5.8 In situ hybridisation

We utilised 4% paraformaldehyde (PFA)-fixed dissected 6 dpf larval brains and followed the protocol described by Thisse & Thisse with slight modifications to perform the in situ hybridisation. The digoxigenin (DIG) RNA labelling kit (Roche Diagnostics, Germany) was used to produce antisense DIG-labelled RNA probes. The specificity of the probes and clones have been described earlier. The *vmat2* probe gave similar signal distribution as characterised earlier for *vmat2*, and the signal was strongly reduced in *vmat2<sup>−/−</sup>* larvae.

The prehybridisation and hybridisation steps were performed at 60°C. Sheep anti-digoxigenin-AP Fab fragments (1:5000; Roche Diagnostics, Germany) were used to detect the in situ hybridisation signals. Staining was performed with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate and samples were incubated at room temperature in the dark. Stained samples were immersed in 80% glycerol, embedded between two cover glasses and analysed under bright-field optics using a Leica DM IRB inverted microscope.
5.9 | Immunohistochemistry

Larvae were killed in ice-cold water at 6 dpf and collected for overnight fixation in 4% 1-ethyl-3,3-(dimethyl-aminopropyl) carbodiimide (EDAC; Carbosynth, Berkshire, UK) or 2% PFA. The detailed protocol for immunohistochemistry and specificity of the antibodies have been described previously.21 Primary antibodies were rabbit anti-histamine 19C (1:5000),64 rabbit anti-5-HT antibody (1:1000; S5545, Sigma, St. Louis, MO, USA),65 anti-tyrosine hydroxylase (TH) monoclonal mouse antibody (1:1000; Product No 22941, Immunostar, Hudson, WI, USA) and anti-HuC/D mouse antibody (1:1000; Invitrogen, Eugene, USA).31 For detection, the samples were incubated with Alexa-conjugated antibodies (Alexa anti-rabbit 488 and anti-mouse 568, Invitrogen, Carlsbad, USA) diluted 1:1000.

For the dopamine staining, zebrafish larvae (6 dpf; vmat2+/+ and vmat2−/−) were fixed by immersion in 5% glutaraldehyde and 1% sodium metabisulfite (MB) in 1X phosphate-buffered saline (PBS; pH 7.4) for 20 hours at 4°C. Brains were dissected out and washed in PBS with 0.3% Triton X-100 and 1% MB (PBST-MB), O/N at 4°C. To increase antibody penetration, we incubated the samples in 0.5 mg/mL collagenase in the following buffer (100 mmol/L Tris base; 1 mmol/L CaCl2; 0.1% Triton X-100; pH 7.4) for 30 minutes at 37 °C, in gentle agitation. After washing in PBST-MB, the samples were pre-treated with 0.5% NaBH4 in deionised water for 1 hour to quench autofluorescence. Following washes in PBST-MB, brains were incubated in blocking solution (4% NGS, 1% DMSO in PBST-MB), O/N at 4 °C. We then incubated the samples with a rabbit polyclonal anti-dopamine antibody (Dr Steinbusch, Maastricht University; 1:750) in blocking solution for 3 days at 4°C. Then, samples were incubated for 1 hour at room temperature with Alexa-conjugated antibodies (Alexa Anti-mouse 568, Invitrogen, Carlsbad, USA) diluted 1:1000 in blocking solution with PBST. Samples were rinsed in PBST and PBS, incubated in increasing glycerol concentrations and mounted in glycerol to RAC. We thank Mr Henri Koivula (BSc) for expert help in fish maintenance. The behavioural and gene expression studies were carried out at the Zebrafish Unit of HiLife Research Infrastructure of the University of Helsinki, which is in part supported by Biocenter Finland.

5.10 | Microscopy and imaging

Brightfield images were taken with a Leica DM IRB inverted microscope with a DFC 480 charge-coupled device camera. Z-stacks were processed with Leica Application Suite software. Immunofluorescence samples were examined using a Leica TCS SP2 AOBS confocal microscope. The Alexa 488- and 568-labelled secondary antibodies were detected using a 488 nm argon laser and a 568 nm diode laser respectively. Emission was detected at 500-550 nm and 560-620 nm respectively. Stacks of images taken at 1.0 μm intervals were compiled, and the maximum intensity projection algorithm was used to produce final images with Leica Confocal software. Cell numbers were counted in each 1.0 μm optical slice using ImageJ 1.52b software (National Institutes of Health, Bethesda, USA). All cell counts were performed by an investigator blinded to the sample type.

5.11 | Data and statistical analysis

Data were analysed by Student’s t test or two-way multiple comparisons ANOVA followed by Sidak's post hoc test, and P < .05 was considered statistically significant. Data analysis was performed by GraphPad Prism version 7 software (San Diego, USA).

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CONFLICT OF INTEREST

The authors declare no competing interest.

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