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Distribution, properties, and inhibitor sensitivity of zebrafish catechol-O-methyl transferases (COMT)

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BCA—bicinchoninic acid (71068)
BCIP—5-bromo-4-chloro-3-indolylphosphate (65409)
CHRM1—cholinergic receptor muscarinic 1
DHBAC—dihydroxybenzylacetic acid (72)
DIG—digoxigenin (15478)
DOPAC—dihydroxyphenylacetic acid (547)
L-DOPA—3,4-dihydroxy-L-phenylalanine (6047)
GAD67—glutamic acid decarboxylase (brain, 67 kDa)
GFAP—glial fibrillary acidic protein
GluN2A—glutamate ionotropic receptor
NMDA type subunit 2 A
GST—glutathione-S-transferase
5-HIAA—5-hydroxyindoleacetic acid (1826)
3-MT—3-methoxytyramine (1669)
NBT—nitro blue tetrazolium chloride (9281)
nNOS—neuronal nitric acid synthase
PFA—paraformaldehyde (712)
Ro41-0960—2'-fluoro-3,4-dihydroxy-5-nitrobenzophenone (3495594)
SAM—S-adenosyl methionine (34756)

Keywords:

COMT
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ABSTRACT

Catechol-O-methyltransferase (COMT; EC 2.1.1.6) is an enzyme with multiple functions in vertebrates. COMT methylates and thus inactivates catecholamine neurotransmitters and metabolizes xenobiotic catechols. Gene polymorphism rs4680 that influences the enzymatic activity of COMT affects cognition and behavior in humans. The zebrafish is widely used as an experimental animal in many areas of biomedical research, but most aspects of COMT function in this species have remained uncharacterized. We hypothesized that both *comt* genes play essential roles in zebrafish. Both *comt-a* and *comt-b* were widely expressed in zebrafish tissues, but their relative abundance varied considerably. Homogenates of zebrafish organs, including the brain, showed enzymatic COMT activity that was the highest in the liver and kidney. Treatment of larval zebrafish with the COMT inhibitor Ro41-0960 shifted the balance of catecholamine metabolic pathways towards increased oxidative metabolism. Whole-body concentrations of dioxyphenylacetic acid (DOPAC), a product of dopamine oxidation, were increased in the inhibitor-treated larvae, although the dopamine levels were unchanged. Thus, COMT is likely to participate in the processing of catecholamine neurotransmitters in the zebrafish, but the inhibition of COMT in larval fish is compensated efficiently and does not have pronounced effects on dopamine levels.

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1. Introduction

Catechol-O-methyltransferase (COMT; EC 2.1.1.6) is an enzyme with diverse functions in the vertebrate organism. The substrates of COMT include xenobiotics with a catechol moiety in the molecule [1], catechol estrogens that act as regulators of cell

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proliferation [2] and carcinogenesis [3], and catecholamine neurotransmitters (dopamine, adrenaline, and noradrenaline) [4].

The COMT gene of humans (and other mammals) encodes two protein forms: the membrane-bound form and the soluble form [6]. Both forms are broadly distributed in the brain and other organs [7]. The human COMT gene polymorphism rs4680 determines an amino acid residue substitution (Val108Met in the soluble protein form) and affects performance in cognitive and working memory tasks [8]. The enzyme form with the Met residue is less stable [9], and COMT activity in postmortem brain samples from Val/Val homozygotes was 38% higher than in Met/Met homozygotes [10].

A homologue of COMT named TOMT or COMT2 was recently identified in humans and rodents [11]. This enzyme can methylate noradrenaline; however, the expression of the gene is restricted to the inner ear, and a recent study demonstrated a methylation-independent function for COMT2 (termed mTOMT) in the auditory system of mice [12]. A gene named COMTD1 (for catechol-O-methyltransferase domain containing 1) has been found on the human chromosome 10 [13] and in other vertebrate species [14], but no reports on the enzyme activity of COMTD1 protein of any vertebrate have been published.

Dopamine inactivation by methylation plays a major role in the prefrontal cortex in the mammalian brain [15,16]. The Val108Met polymorphism has been extensively studied as a risk factor for psychiatric disorders, including schizophrenia: however, an association with genotype and disease susceptibility has not been proven conclusively [17], although genetic variation in the COMT gene affected certain behavioral traits in schizophrenia patients [18], often in an estrogen-dependent manner [19]. Variation of COMT activity affects gene expression in dorsolateral prefrontal cortex (*GluN2A* and *GAD67* [20]; *CHRM1* [21]) and neuroanatomical features of the brain, as shown in human studies of dopaminergic cell populations in substantia nigra [22], cingulate cortex volume [23], and white and gray matter density in the whole brain [24], as well as in animal experiments [25]. Another important function of COMT in the nervous system is related to pain processing [5,26,27]. Certain functions of COMT in peripheral tissues are of medical relevance as well. Methylation of dopamine by COMT in the kidney plays a key role in the regulation of natriuresis, as shown by experiments in rats [4]. Low levels of COMT activity increase the risk of preeclampsia, a severe disorder of pregnancy, due to insufficient synthesis of 2-methylestradiol and the related disturbances in angiotensin signaling [28,29].

The major area of therapeutic use of COMT inhibitors is the treatment of Parkinson's disease (PD). Inhibitors that do not penetrate the blood–brain barrier are used to increase the bioavailability of L-dioxyphenylalanine (L-DOPA), a dopamine precursor drug [30]. COMT inhibition also prevents pathological accumulation of homocysteine formed during the methylation of L-DOPA by COMT and the associated development of peripheral neuropathy [31].

Genetically modified mice that are fully devoid of COMT [32] or synthesize the membrane-bound isoform [33] or the soluble isoform only [34], as well as animals that express the human “Met-COMT” or “Val-COMT” [5,35], have been produced and characterized. The animals were viable and lacked gross morphological abnormalities; however, slight but distinct differences in behavior [32–35], brain anatomy [25] and the development of the enteric nervous system (namely, elevated GFAP immunoreactivity in myenteric ganglia, reduced density of acetylcholinesterase-immunoreactive fibers, and altered distribution of nNOS-immunopositive neurons in knockout mice) [36] were identified. Changes in non-neural tissues of COMT knockout mice concerned the development of T-, B- and NK-cell populations, especially in male animals [37].

COMT enzyme activity, immunoreactivity, and/or expression of COMT genes was reported for a number of fish species. COMT enzyme activity was detected in the brain of African catfish, with especially high activity observed in the nucleus of the posterior recess of the diencephalon, the tectum opticum, and the torus semicircularis [38]. The *comt* gene of stinging catfish was expressed in multiple tissues, including the brain, liver, and ovary, where the enzyme regulated egg development through inactivating catechol estrogens [39]. The *comt* gene of sea bass was apparently involved in immune response, since the expression of this gene in fish spleen was significantly downregulated upon vaccination and bacterial challenge [40]. COMT enzymatic activity was detected in trout gills, and the gills were supposed to take part in the inactivation of circulating catecholamine neurotransmitters [41]. Neither functional sequence polymorphisms in fish COMT proteins nor the existence of membrane-bound and soluble COMT enzymes encoded by the same fish gene has been reported by now.

Zebrafish are widely used as experimental animals in biomedical research, including neurobiology. The basic structure of the zebrafish brain and the neurotransmitters found in the CNS are similar to those of mammals [42]. Both COMT genes found in the zebrafish genome (*comt-a* and *comt-b*) are expressed, as shown by RT-PCR analysis of zebrafish cDNA libraries [42]. GST-tagged COMT-a protein produced in a recombinant system was enzymatically active in vitro [43]. Inactivation of xenobiotics has been proposed as the primary function of the zebrafish COMT [43]. However, zebrafish COMT enzymes are probably also involved in the processing of catecholamine neurotransmitters, since expression in the brain and stress-related changes of the expression levels have been reported for both *comt-a* [44] and *comt-b* [45]. The inhibition of monoamine oxidase, an enzyme that acts along with COMT to inactivate catecholamine neurotransmitters, did not have an effect on dopamine levels in whole zebrafish larvae, this probably being due to the existence of alternative pathway(s) of dopamine degradation. The product of dopamine methylation by COMT, 3-methoxytyramine (3-MT), was detected in larval zebrafish [46], and therefore the presence of COMT enzyme activity in this species could be expected. A proteomic study of zebrafish synapse proteins revealed the presence of both COMT proteins (as well as MAO) in the synaptosome fractions, and COMT-b was found in the postsynaptic density fraction as well, although in smaller quantities [47]. However, the tissue distribution of COMT activity and *comt* gene expression, as well as the effects of COMT inhibitors on zebrafish, have not been characterized yet.

We hypothesized that organs that perform the inactivation of catecholamines or xenobiotic catechols, such as the liver, gut, gills, kidney and the CNS express both forms of *comt* and display COMT activity. The aim of the present study was thus to characterize the basic features of zebrafish COMTs, such as the quantitative and qualitative patterns of distribution of *comt* mRNAs and enzymatic COMT activity, as well as the effects of COMT inhibition on neurotransmitter metabolism in larval fish.

2. Materials and methods

2.1. Fish

Zebrafish of the Turku line, maintained in our laboratory for more than fifteen years ([48,49]), were used in the present work. The permits for the experiments were obtained from the Office of the Regional Government of Southern Finland. The fish were housed at 28.5 °C in E3 embryo medium (larval fish) or in an aquarium system from Aquatic Habitats (United States) with circulating water controlled for temperature, pH, and conductivity and disinfected by UV illumination. The adult fish were fed twice daily with

SDS-400 flake food (Scientific Diets Services, Witham, England) and artemia, and the larval fish older than 5 days were fed with SDS-100 flake food (Scientific Diets Services).

2.2. Chemicals

All chemicals were purchased from Sigma-Aldrich (Buchs, Switzerland) if not stated otherwise. Deionized formamide used for *in situ* RNA hybridization was from VWR (Radnor, PA, USA). Ethanol was from Altia (Rajamäki, Finland). Water used in the experiments was purified by a MilliQ device (Millipore, Billerica, MA, USA).

2.3. Cloning of fragments of *comt-a* and *comt-b* coding sequences

Fragments of *comt-a* and *comt-b* coding sequences were amplified from a 5-dpf larval zebrafish cDNA library with the following primers (synthesized by Oligomer Oy, Helsinki, Finland):

comt-a-forward TGCTGGCGCTTCTCTGGCAC;
comt-a-reverse AGAAGACCGATTCTCCAGGCCG
comt-b-forward TTGCGTCTCGCAGGTTTAACAGAAGG;
comt-b-reverse ATACAGAGCTTCCAAGCCATCTCTT.

The PCR products were purified and cloned into pGEM-T-easy. The structure of the cloned fragments was verified by sequencing. The constructs prepared were named “pGEM-T-easy-*comt-a*” and “pGEM-T-easy-*comt-b*”.

2.4. In situ RNA hybridization (ISH)

The plasmids “pGEM-T-easy-*comt-a*” and “pGEM-T-easy-*comt-b*” were linearized with restriction enzymes listed in Table 1 (2 µg plasmid, 10 U enzyme, 2 h at 37 °C), purified with a MinElute Reaction Cleanup Kit (Qiagen, Hilden, Germany), and used as templates for riboprobe synthesis.

DIG-labeled riboprobes were synthesized according to the protocol recommended by the DIG RNA labeling kit manufacturer (Roche, Mannheim, Germany). The reaction mix contained the template, DIG RNA labeling mix, reaction buffer, RNA polymerase T7 or SP6 (listed in Table 1 for specific probes), and RiboLock RNase inhibitor. The reaction mix was incubated for 2 h at 37 °C. The probes were precipitated overnight at –20 °C in the presence of LiCl and ethanol, washed twice with ice-cold 70% ethanol, air-dried, re-dissolved in hybridization mix for ISH, and stored at –80 °C. ISH was performed according to the protocol in [50] with NBT/BCIP detection as shown previously [49].

Three-month old zebrafish (three males and three females) were killed in ice-cold water and immersion fixed in 4% paraformaldehyde (PFA). The specimens were incubated overnight in a solution containing 20% sucrose and 0.5 M EDTA, and 40 µm thick sections were cut on a Leica cryostat and thaw-mounted on Superfrost glass slides (VWR). ISH on sections was performed in an In Situ Pro VSI device (Intavis, Cologne, Germany) at a hybridization temperature of 65 °C. Hybridization with sense RNA probes was used to control staining specificity.

2.5. Quantitative PCR

Organs and tissue samples were dissected from four 8-month-old male zebrafish, immediately frozen in liquid nitrogen, and stored at –80 °C until analysis. The gastrointestinal tract was divided into the anterior part and the posterior part upon dissection (referred to as “anterior” and “posterior” gut). The Qiagen kit was used for RNA isolation as recommended by the manufacturer, and 100 ng RNA (quantified using spectrophotometry) was used for cDNA synthesis with random hexanucleotide primers. Reverse transcription was performed using SuperScript™ III reverse transcriptase (Invitrogen) according to instructions provided by the manufacturer (reaction volume was 20 µl, and the reverse transcription was carried out at 55 °C for 1 h). The qPCR analysis was carried out using a Roche LightCycler device and SYBR Green Master Mix reagent supplied by Roche according to instructions provided by the manufacturer. The primers for *comt-a* qPCR were as in [44], and those for *comt-b* qPCR were as in [45]. Thermocycling parameters were as follows: 95 °C for 5 min and 45 cycles of the following: 95 °C for 10 s, 60 °C for 15 s, and 72 °C for 20 s. Fluorescence changes were monitored with SYBR Green after every cycle, and dissociation curve analysis (increase from 60 °C to 95 °C at 0.1 °C/s with continuous fluorescence readings) was performed after the last amplification cycle to ensure that only a single amplicon was obtained. All reactions were performed in duplicate. LightCycler 480 software was used to calculate the results, and the data were processed by the comparative method using C_t values for the product of the *efla* gene as the reference values. Primers 5'-CCAACTTCAACGCTCAGGTCA-3' and 5'-CAAACCTGCAGGCGATGTGA-3' were used for the reference gene *efla*.

2.6. COMT activity assays

Four adult (1-year old) male zebrafish were killed by spinal cord transection after terminal anesthesia by immersion in ice-cold water. The brains, other organs, and muscle tissue samples were dissected and stored at –80 °C until analysis; the gastrointestinal tract (referred to as “gut”) was analyzed as a whole. The samples were homogenized on ice in 0.1 M phosphate buffer (PB) and centrifuged for 10 min at 4 °C. Aliquots of the supernatant (usually 100 µl) were added to 150 µl of a solution of the substrate dihydroxybenzylacetic acid (DHBAC) and cofactors S-adenosyl methionine (SAM) and 5 mM MgCl₂ in phosphate buffer, pH 7.4. The final concentrations of the reagents were 200 µM DHBAC, 200 µM SAM, and 5 mM MgCl₂. The samples were incubated for 30 or 60 min at 37 °C and then the reaction was stopped with 25 µl ice-cold 4 M perchloric acid. The resulting mixtures were centrifuged for 10 min at 4 °C, and 20 µl of supernatant were injected into the HPLC system. Concentrations of vanillic and isovanillic acid, the two products of DHBAC methylation, were calculated from standard curves obtained by injecting solutions of the pure compounds into the HPLC system. Protein concentration in the samples was measured using the Pierce® BCA Protein Assay Kit (Thermo Fisher Scientific Inc., Rockford, IL, USA), and the concentration of reaction products was normalized for the concentration of protein. The assays were performed in triplicate.

Table 1

Enzymes used for plasmid linearization and in vitro transcription for the synthesis of *comt* riboprobes.

Plasmid	Antisense riboprobe synthesis		Sense riboprobe synthesis	
	Restrictase	Transcriptase	Restrictase	Transcriptase
pGEM-T-easy- <i>comt-a</i>	SpeI	T7	NcoI	SP6
pGEM-T-easy- <i>comt-b</i>	NcoI	SP6	SpeI	T7

2.7. Treatment of animals with COMT inhibitor

COMT inhibitor Ro41-0960 (Sigma, USA) was dissolved in DMSO at 10 mM and stored at -20°C . Stock solution of the inhibitor was added to the wells of 6-well plates where the fish were kept ($n = 4$ groups of 30 fish per treatment condition for the treatment from 1 dpf till 3 or 5 dpf; $n = 3$ groups of 30 fish per treatment condition in the case of short-term treatment of 14 dpf fish). DMSO was added to the control wells. The medium was exchanged daily if the treatment continued for more than one day. The larvae were killed on ice and homogenized by sonication on ice in 150 μl 2% perchloric acid. The homogenates were centrifuged and analyzed by HPLC.

2.8. Measurement of catecholamine levels using HPLC

The analyses for the COMT activity assay and the quantitation of catecholamine neurotransmitters and metabolites thereof were performed on a high-performance liquid chromatography (HPLC) system equipped with a Waters Concorde electrochemical detector set to a potential +0.80 V, column oven, and a column Gemini C18 5 μm 150 $\times$ 4.60 mm (Phenomenex, Torrance, CA, USA). The mobile phase consisted of purified water with 8% methanol, 50 mM citric acid, 1.5 mM 1-octanesulfonic acid, 0.05 mM EDTA, and 50 mM phosphoric acid. The column temperature was set at 37°C and the flow rate at 1 ml/min. System control, data acquisition and analysis employed the Waters Empower software (Waters, Milford, MA). Standard curves for the substances analyzed were linear from 10 nM to 1 μM . The procedure has been described in detail [46].

2.9. Bioinformatics and statistical analysis

Information on COMT genes from different vertebrate species was retrieved from the Ensembl database (release 85 [14]). Synteny database [51] was used to assess the syntenic relationships between human and zebrafish COMT genes with the following settings: source genome *Danio rerio*, outgroup *Homo sapiens*, sliding window size 100 genes. Phobius software [52] was used to test for the presence of transmembrane sequences in zebrafish COMT proteins.

The results of COMT activity assays and HPLC measurements of neurotransmitter levels were analyzed using one-way ANOVA followed by Tukey's post hoc test in OriginPro 8.6 software (OriginLab Corporation, Northampton, MA, USA); normal distribution of the values and equal variance of the samples were tested in all cases.

3. Results

3.1. Structure of *comt* genes and COMT proteins in the zebrafish

The fragment of the zebrafish chromosome 8 harboring the *comt-a* gene was syntenic to the fragment of the human

chromosome 22 harboring the COMT gene (Fig. 1), whereas there was no syntenic relationship between the zebrafish *comt-b* gene (located on chromosome 11) and the COMT genes of other vertebrates available for comparison in the Synteny database. No homologues of the human leucine-rich transmembrane O-methyl transferase (*LR-TOMT*) gene (also known as *COMT2*) that encodes a protein with catechol-O-methyl transferase activity [11] were identified in the zebrafish genome. Interestingly, other teleost fish species with genome data available on Ensembl (medaka, stickleback, and fugu) presumably have a single COMT gene and a single *LR-TOMT* gene, whereas the zebrafish has two COMT genes and no *LR-TOMT* gene.

The hydrophobic N-terminal fragments of the predicted amino acid sequence of zebrafish COMT proteins (underlined in Fig. 2) can serve as membrane anchors or as signal peptides of secreted proteins. The human COMT gene is known to give rise to two protein isoforms, the soluble one and the membrane-attached one, due to alternative initiation of translation. Neither of the zebrafish COMT sequences contained a methionine residue that could serve as the N-terminal residue for a protein isoform arising from alternative initiation. Analysis of COMT sequences by the Phobius software that can discriminate between putative signal peptides and putative membrane anchors showed that the COMT-b protein of the zebrafish may be membrane-anchored, whereas COMT-a is most likely soluble and may be secreted (since the protein sequence contains a putative signal peptide).

The degree of sequence identity to human COMT was 56% for both putative COMT proteins of the zebrafish. Amino acid residues involved in substrate and cofactor binding [53] were conserved in both COMT proteins of the fish, except for Ile141 of human COMT that was replaced by a phenylalanine residue in zebrafish enzymes. However, the interaction of the cofactor S-adenosyl methionine (SAM) with this residue involves the amide nitrogen rather than the side chain (Fig. 2), and therefore the substitution should not have a dramatic effect on enzyme properties.

3.2. COMT expression and activity patterns

Quantitative RT-PCR revealed the expression of both *comt-a* and *comt-b* genes in multiple organs and tissues of the adult zebrafish (Fig. 3). The expression level of *comt-b* (normalized to the expression of the housekeeping gene *efla*) was generally higher than that of *comt-a*, with the exceptions of the gill and gut. The expression level of *comt-a* was the highest in the liver, whereas *comt-b* was expressed at high levels in the central nervous system, eye, liver and kidney. Expression of both genes in the heart and muscle tissue was the lowest.

In situ RNA hybridization on axial sections of juvenile (three-month old) fish revealed the presence of *comt-a* mRNA in all cell layers of the retina (Fig. 4A, arrows and line), with especially high expression in the inner nuclear layer (Fig. 4A, line). Expression in the brain was the highest in the area close to the midline of the

Fig. 1. Synteny between the fragments of zebrafish chromosome 8 and human chromosome 22 harboring catechol-O-methyltransferase genes.

Fig. 2. Sequence alignment of COMT proteins from human (P21964), mouse (O88587), and zebrafish (A-F4ZGF2, B-A4IG53). UniProt sequence IDs are shown in brackets. Methionine residues that are the N-terminal residues in the soluble isoforms of mammalian COMT (Met51 in the human sequence and Met44 in the mouse sequence) are underlined. Amino acid residues involved in magnesium binding are marked with "m", those involved in S-adenosyl methionine binding are marked with "s", and those involved in catechol substrate binding are marked with "c". The N-terminal hydrophobic fragments that can serve as signal peptides or membrane anchors in zebrafish proteins are underlined.

Fig. 3. Expression levels of *comt-a* (A) and *comt-b* (B) genes normalized by the expression level of *efla* in organs and tissues of adult zebrafish (n = 4).

telencephalon (Fig. 4 A, outlined), the periventricular gray zone (PGZ) of the optic tectum, the area around the walls of the diencephalic ventricle in the hypothalamus, and the preglomerular nucleus (Fig. 4B, dashed outline, solid outline, and asterisks respectively).

The gene was also expressed in the ovary (Fig. 4C, dashed outline), the spinal cord (Fig. 4C and E, white outlines with

arrowheads), the liver (Fig. 4E, black dashed outline), and the gut (Fig. 4C, black outline, and Fig. 4E, thick white outline). Hybridization with the sense riboprobe used as control did not show any signal comparable with that obtained with the antisense riboprobe (Fig. 4D, PGZ is outlined, compare to PGZ staining pattern in 4B). There was virtually no *comt-a* mRNA signal in the gut epithelium, while the lamina propria directly adjacent to the gut epithelium

Fig. 4. In situ RNA hybridization for *comt-a* on axial (A–F) and horizontal (G, H) sections of 3-month-old zebrafish. A–*comt-a* mRNA in the medial part of the telencephalon (outlined) and layers of the retina (black arrow-granular cell layer, white line-inner nuclear layer, white arrowhead-outer nuclear layer); B–*comt-a* expression domains in the periventricular gray zone (dashed white line) and around the diencephalic ventricle (solid white line) of the brain; C–*comt-a* mRNA in the spinal cord (arrowhead, solid white outline), ovary (dashed white outline), and gut (solid black outline); D–hybridization with a sense probe (control, periventricular gray zone (labeled PGZ) outlined for comparison with B; DiV label shows the position of the diencephalic ventricle); E–*comt-a* mRNA in the spinal cord (arrowhead, solid white outline), kidney (thick dashed white line), liver (dashed black line), and gut (white line); F–*comt-a* mRNA in the gut (the border of lamina propria is shown by a white line); G–*comt-a* mRNA in the kidney (the anterior part of the kidney is outlined); H–*comt-a* mRNA in the testis. Scale bar, 1 mm in A–D and E–H, 0.1 mm in F.

showed a strong expression, with the *comt*-expressing cells forming a nearly continuous layer (Fig. 4C, E, and high-magnification image in Fig. 4F with the outer border of the lamina propria shown by the line). The expression level was high in the kidney (Fig. 4E, thick dashed white outline), and especially in its anterior part, as evident from hybridization to a horizontal section (Fig. 4G, outline). The expression of *comt-a* was also detected in the testis (Fig. 4H).

The expression pattern of *comt-b* was largely similar to that of *comt-a*, with expression detected in the retina (Fig. 5A, arrowhead) and the central part of the telencephalon (Fig. 5A, outlined), periventricular gray zone of the optic tectum (Fig. 5B, dashed outline; Fig. 5C, solid outline), cells adjacent to the diencephalic ventricle in the hypothalamus (Fig. 5B, solid outline), the periglomerular nucleus (Fig. 5B, asterisks) and the olfactory bulb (Fig. 5C, arrow) in the brain. Expression outside the CNS was

Fig. 5. In situ RNA hybridization for *comt-b* on axial (A, B, D) and horizontal (C, E) sections of 3-month old zebrafish. A—*comt-b* mRNA in the telencephalon (outlined) and the retina (arrowhead); B—*comt-b* expression domains in the periventricular gray zone (dashed white line) and around the diencephalic ventricle (solid white line) of the brain; C—*comt-b* expression domains in the brain (periventricular gray zone is outlined) and retina (arrowhead); D—*comt-b* mRNA in the spinal cord (dashed outline), esophageal epithelium (arrowhead), and gills (outlined); E—hybridization with a sense probe used as control: note the absence of signal in the retina (arrowhead) and the gills (outlined). Scale bar, 1 mm.

observed in the gills (Fig. 5D, outline), especially the parts of gill filaments adjacent to the gill arch, and in the esophageal epithelium (Fig. 5D, arrowhead). There was no signal in the samples hybridized with the sense riboprobe (Fig. 5E, compare the retina indicated by the arrowhead to Fig. 5C and the outlined gills to Fig. 5D).

COMT activity normalized by protein content was the highest in the liver (significantly higher than in all other tissues) and kidney (significantly higher than in all other tissues except for the liver) and substantial in the brain and gut. Activity in muscle, whole eyes, and gill was the lowest (Fig. 6A). The ratio between the amounts of isomeric products of dihydroxybenzylacetic acid (DHBAC) methylation (vanillic and isovanillic acid) formed by COMTs from different organ homogenates varied to a certain extent (Fig. 6B); however, only COMT activity from the gut differed significantly from others with regard to spatial selectivity ($p < 0.05$ in pairwise comparisons).

3.3. COMT inhibition affects the balance of metabolic pathways

COMT enzymatic activity in homogenates of larval zebrafish and adult zebrafish liver was completely suppressed by 1 μ M COMT inhibitor Ro41-0960: neither vanillic nor isovanillic acid was detected by HPLC in reaction mixtures prepared according to the standard assay procedure. Inhibitor concentration used in vivo was adjusted using the results of preliminary experiments:

36 hpf larvae exposed to 2 μ M inhibitor or more died within a few hours, whereas 5 dpf larvae could tolerate 10 μ M inhibitor without any apparent changes in locomotor activity, positioning in the water, or pigmentation. The death of fish exposed to high doses of the inhibitor was preceded by hypomotility and a drastic reduction of heart rate.

Treatment of 14 dpf larval fish with 10 μ M Ro41-0960 for 2 h, as well as prolonged treatment of developing larvae (from 24 hpf to 3 or 5 dpf) induced a significant increase in the whole-body concentration of DOPAC (Table 1), a product of dopamine oxidation by MAO (which is further metabolized by COMT into homovanillic acid). There were no significant changes in dopamine concentration. Interestingly, the concentration of 5-HIAA, a product of oxidative metabolism of serotonin, was increased after exposure to COMT inhibitor as well (Table 2), and this may be indicative of an increase in MAO activity after COMT inhibition.

4. Discussion

The expression of *comt-a* and *comt-b* genes and enzymatic activity of COMT were detected in all organs of the zebrafish where inactivation of catecholamines or xenobiotic catechols could take place, but the relative abundance was highly variable. *Comt-a* was the predominant form in the gut, gills and spleen, and *comt-b* mRNA expression was more abundant in the liver, brain

Fig. 6. COMT activity in zebrafish tissue homogenates (measured with DHBAC as substrate, $n = 4$) (A) and spatial selectivity of COMT in different tissues (the relative abundance of vanillic acid, the product of meta-methylation, among the products of DHBAC methylation, $n = 4$) (B). The dashed lines in (B) show the values for rat COMT isoforms cited from (Nissinen et al., 1984) for comparison. "Brain (juv.)" – brains of 3-month old (juvenile) male zebrafish, "brain (adult)" – brains of 1.5-year old male zebrafish.

Table 2
Levels of DOPAC and 5-HIAA in zebrafish after COMT inhibitor treatment (mean $\pm$ SD; $n = 4$ groups of 30 fish per treatment condition for the treatment from 1 dpf till 3 or 5 dpf; $n = 3$ groups of 30 fish per treatment condition in the case of short-term treatment of 14 dpf fish).

Fish age and inhibitor concentrations used for the treatment	[DOPAC], pmol/mg protein		[5-HIAA], pmol/mg protein	
	Control fish	Treated fish	Control fish	Treated fish
3 dpf; from 2 μ M at 24 hpf to 10 μ M at 3 dpf	0.19 $\pm$ 0.03	3.45 $\pm$ 0.29 ($p < 0.001$)	18.5 $\pm$ 2.04	23.1 $\pm$ 1.7 ($p < 0.05$)
5 dpf; from 2 μ M at 24 hpf to 10 μ M at 5 dpf	0.37 $\pm$ 0.04	1.24 $\pm$ 0.04 ($p < 0.0001$)	14.6 $\pm$ 0.97	25.6 $\pm$ 0.98 ($p < 0.0001$)
14 dpf; 10 μ M for 2 h	0.49 $\pm$ 0.05	2.21 $\pm$ 0.15 ($p < 0.001$)	4.3 $\pm$ 0.24	5.1 $\pm$ 0.28 ($p < 0.05$)

and kidney. COMT inhibition in larval fish did not cause an elevation of dopamine levels, but the intensity of oxidative metabolism of dopamine and serotonin increased, probably due to compensatory neurochemical changes.

The genomic fragment harboring the *comt-a* gene of the zebrafish was syntenic to the chromosomal fragment harboring the

human *COMT* gene, but there was no syntenic relationship between the human gene and the zebrafish *comt-b* gene. This may be indicative of the emergence of *comt-b* in a genomic duplication event.

No homologues of the *lr-tomt* gene (leucine-rich transmembrane O-methyl transferase, termed COMT2 in humans and

mTOMT in mouse [11]) were found in the current genome assembly for zebrafish, although the genomes of other fish species listed in the Ensembl database contained a single gene for COMT and a single gene for LR-TOMT.

Membrane anchoring was predicted for zebrafish *comt-b* by the Phobius software, whereas *comt-a* is probably a secreted protein. The predicted protein sequences of both zebrafish COMTs did not contain Met (or Val) residues that could serve as the N-terminal residues of shorter protein isoforms. Therefore, neither of zebrafish COMT genes is expected to give rise to two enzyme forms, in contrast to the mammalian genes. The expression patterns of *comt-a* and *comt-b* in zebrafish were quite similar: notably, the tissue distribution patterns of soluble and membrane-bound COMT isoforms in mice are similar as well [7].

The products of zebrafish genes *comt-a* and *comt-b* are very likely to have COMT activity, since the protein sequences show high overall similarity to mammalian COMT sequences and the key residues involved in catalysis are conserved. A fusion protein of GST and zebrafish COMT-a showed enzymatic activity in vitro, although activity of the protein with the GST moiety removed was low and poorly reproducible [43].

The levels of COMT activity and the abundance of *comt* mRNAs in the various organs of zebrafish were generally in good agreement, with the highest activity and expression levels in the liver and very low activity and expression in muscle. Discrepancy between the relative levels of COMT activity and gene expression (for example, in case of the eyes) may be related to the fact that enzyme activity was normalized to total protein content in the tissues, while the mRNA levels were normalized to the expression level of the *efla* gene. High abundance of RNases in the liver tissue and slow penetration of the fixative through skin and muscle may lead to partial degradation of COMT mRNAs and thus account for the relatively weak ISH signal in the liver on whole-fish sections.

Zebrafish brain homogenates showed COMT activity, and expression of *comt* genes was detected in several areas of the zebrafish brain: the olfactory bulb, the areas near the telencephalic and diencephalic ventricles, the periventricular gray zone of the diencephalon, and the periglomerular nucleus. Inactivation of catecholamine neurotransmitters is likely to occur in these brain areas because they receive catecholaminergic innervation. Indeed, the olfactory bulb of the zebrafish contains a prominent dopaminergic cell population [48], but the expression level of the *mao* gene and the level of MAO enzymatic activity in this area is low [46]. A recent study showed that COMT made a substantial contribution to dopamine processing in the olfactory bulb of the mouse [54]. The dopamine receptor 2a (*drd2a*) gene is expressed in the periglomerular nucleus of the adult zebrafish [55], the mRNA [56] and protein [57] for α_2A -adrenoceptor are found in the medial part of the dorsal telencephalon, and β -adrenoceptor immunoreactivity is found in the zebrafish PGZ [58]. Therefore, COMT may participate in neurotransmitter metabolism in all these brain areas, along with MAO. The expression of zebrafish *comt* genes in the zones of the diencephalon adjacent to the ventricles was reminiscent of the distribution of COMT immunoreactivity in African catfish brain [38]. COMT immunoreactivity was also detected in the circumventricular areas of the rat brain [59].

COMT immunoreactivity in the rat retina was confined to the ganglion cell layer [59], and thus had a more restricted pattern than *comt* gene expression in the zebrafish retina. Dopamine signaling plays an important role in functioning of the zebrafish eye [60], and the contribution of COMT to dopamine processing in the retina is likely. Methylation of DOPAC to form HVA was detected in the mouse retina [61].

In contrast to the findings of COMT immunoreactivity in the gut epithelium of rats and mice [59,62], zebrafish *comt* genes were not expressed in the epithelium, but were abundantly expressed in the

lamina propria, and to some extent, in the microvilli (mouse COMT protein was also found in the microvilli of the intestine [62] and in intestinal macrophages). The presence of a continuous layer of *comt*-expressing cells in the zebrafish gut is in good agreement with the proposed role of COMT in the inactivation of dietary catechols [43]: *comt*-expressing cells probably form a “barrier” beneath the gut epithelium.

The presence of COMT activity and the expression of *comt* genes in the zebrafish gills is in agreement with findings in the trout [41]. Mouse lungs are poor in COMT [7]; however, the fish gills fulfill a secretory function in addition to a function in oxygen exchange, and this may account for the difference in relative expression levels between species.

The expression of *comt* genes in the zebrafish ovary is in agreement with the findings in catfish [39] and is probably indicative of the role of zebrafish COMT in the methylation of catechol estrogens.

Zebrafish spleen is likely to be the target of adrenergic regulation, similar to that reported in other fish [63], but MAO content in this organ is very low [64] and therefore COMT may be required for the inactivation of the neurotransmitters involved in adrenergic signaling. Moreover, zebrafish COMT present in the spleen may be involved in the regulation of immune response, as reported for mice [37] and fish (sea bass) [40]. COMT protein is present in the spleen of mice [7] and rats [59].

The zebrafish kidney showed *comt* gene expression and rather high COMT enzyme activity, similarly to the kidneys of other fish [40] and mammals [4]. The functions of COMT in the kidney may include both the regulation of dopamine levels to control salt homeostasis, as demonstrated for rats [4], and the degradation of excess adrenaline synthesized by scattered cells in the anterior part of the kidney [65] that serve as the adrenal glands in teleost fish.

The contribution of COMT and MAO to dopamine inactivation can be inferred from the levels of dopamine and its metabolites (3-MT, the product of methylation, and DOPAC, the oxidation product) in the normal tissue. The levels of both DOPAC and 3-MT in the mouse striatum [66] and in the entire right hemisphere [67] were lower than the levels of dopamine, and the levels of 3-MT were lower than the levels of DOPAC. The levels of 3-MT reportedly were the same or higher than the levels of DOPAC in most part of the chick brain (except the optic lobes and the cerebellum) [68], and in all regions of the trout brain [69]. However, the findings reported in [69] were attributed to the post-mortem increase of 3-MT levels, since the levels of dopamine metabolites usually did not exceed 20% of the dopamine levels in the brains of rainbow trout, goldfish, and other teleost fish species [70].

Interestingly, 3-MT, a product of dopamine methylation by COMT, elicited a strong olfactory response in goldfish [71], and therefore COMT activity might play a role in olfactory communication in zebrafish.

Levels of the products of oxidative metabolism of dopamine, noradrenaline and adrenaline in human blood plasma were elevated after administration of the peripherally acting COMT inhibitor entacapone, but the concentrations of noradrenaline and adrenaline remain unchanged [72]. Thus, the shift in the balance of catecholamine degradation pathways that compensated for COMT inhibition was observed in humans as well.

Taken together, this study suggests that the distribution of COMT enzymatic activity and gene expression in zebrafish has many common features with patterns observed in other vertebrates, including mammals. Therefore, pharmacological experiments and genetic modification in zebrafish can provide highly relevant information on the functional role of COMT in vertebrates. However, the transmembrane and soluble COMT forms may be encoded by different genes in the zebrafish, and this should be

taken into account as experiments are planned and interpreted. The more abundant expression of *comt b* in the CNS suggests that this form is more important in metabolism of neuronal catecholamines.

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