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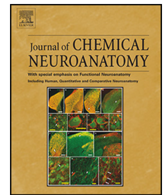

Localization of genes encoding metallothionein-like protein (*mt2* and *smtb*) in the brain of zebrafish

Seong Lin Teoh, Satoshi Ogawa, Ishwar S. Parhar *

Brain Research Institute, School of Medicine and Health Sciences, Monash University Malaysia, Bandar Sunway 47500, Selangor, Malaysia

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ABSTRACT

Metallothionein (MT) is a small cysteine-rich heavy metal-binding protein involved in metal homeostasis, detoxification and free radical-scavenging. MT is ubiquitously expressed in several tissues, but its role in the central nervous system is not well understood. In this study, we identified two MT homologous genes (*mt2* and *smtb*) in the zebrafish. Digoxigenin-*in situ* hybridization showed the expression of *mt2* and *smtb* genes in the ventricular layers in the telencephalon, diencephalon, mesencephalon and rhombencephalon, most of which are cell proliferating regions in the brain of zebrafish. Cellular characteristics of MT genes expressing cells were examined by double-labelling with markers for neurons (HuC/D) and astrocytes (glial fibrillary acidic protein, GFAP and S100 protein) and cell proliferation marker (PCNA). *mt2* and *smtb* mRNAs are expressed in neurons and not in astrocytes, and they were co-localized with PCNA. These results suggest that *mt2* and *smtb* may play an important role in neurogenesis and neuroprotection.

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1. Introduction

Metallothionein (MT) is a versatile low molecular mass (6–7 kDa), non-enzymatic, heat-stable and heavy metal-binding intracellular protein. MT was first isolated from the horse kidney cortex as a protein which binds to cadmium (Margoshes and Vallee, 1957). MTs are composed of a single polypeptide chain of 61–68 amino acids, 16–20 of which are cysteine (Cys), and there is an absence of disulfides, aromatic amino acids and histidines (Hidalgo et al., 2001; West et al., 2008). A characteristic feature of the MT amino acid sequence is the presence of Cys-Cys, Cys-X-Cys and Cys-X-Y-Cys motifs where X and Y are amino acids other than Cys (West et al., 2008). The high Cys content enables MT to interact with metalic ions (Ambjorn et al., 2008).

Four isoforms of MT namely MT1, 2, 3, and 4 have been identified in mammalian species. In humans, the MT genes are tightly clustered in the q13 region of chromosome 16, consisting of nine *MT1* genes designated *MT1A*, *-B*, *-E*, *-F*, *-G*, *-H*, *-L*, *-M* and *-X*, and a single gene encoding *MT2*, *-3*, and *-4* (West et al., 2008). In non-mammalian species, avian species possess two MT isoforms (MT1 and MT2), whereas reptiles and amphibians possess only a single MT (Trinchella et al., 2012). Two MT isoforms (MT1 and MT2 which are also designated as MTA and MTB) have been identified in several teleost species (Wu et al., 2008; Gao et al., 2009). In the Javanese medaka (*Oryzias javanicus*) and Mozambique tilapia (*Oreochromis mossambicus*), only a single MT has been identified (Wu et al., 2008; Woo et al., 2006). In the zebrafish (*Danio rerio*), in addition to two MT isoforms (*mt1* and*mt2*), a third *mt* gene with similar features to *mtb* (termed "similar to *mtb*" = *smtb*) has been identified (Wu et al., 2008).

Abbreviations: C, central canal; CC, cerebellar corpus; CM, mammillary body; Cven, ventral rhombencephalic commissure; Cys, cysteine; Dc, central zone of dorsal telencephalic area; DIG, digoxigenin; DIL, diffuse nucleus of the inferior lobe; DiV, diencephalic ventricle; Dl, lateral zone of dorsal telencephalic area; Dm, medial zone of dorsal telencephalic area; EAE, experimental autoimmune encephalomyelitis; GC, central gray; GFAP, glial fibrillary acidic protein; Had, dorsal habenular nucleus; Hav, ventral habenular nucleus; Hb, habenula; Hd, dorsal zone of the periventricular hypothalamus; IRF, inferior reticular formation; ISH, *in situ* hybridization; LCa, caudal lobe; LR, lateral recess of diencephalic ventricle; LRP, low density lipoprotein receptor-related protein; MFN, medial funicular nucleus; MLF, medial longitudinal fascicle; MT, metallothionein; NMLF, nucleus of medial longitudinal fascicle; NIII, nucleus of oculomotor nerve; NIV, nucleus of trochlear nerve; NXm, vagal motor nucleus; OT, optic tectum; PCNA, proliferative cell nuclear antigen; PGZ, periventricular gray zone of the optic tectum; Pit, pituitary; RVe, rhombencephalic ventricle; *smtb*, similar to *mtb*; Spred-2, sprout-related EVH1 domain containing protein 2; SRF, superior reticular formation; TBS, bulbo-spinal tract; TeV, tectal ventricle; TPp, periventricular nucleus of posterior tuberculum; TSA, tyramide signal amplification; VCl, lateral lobe of the cerebellar valvula; VCm, medial lobe of the cerebellar valvula; Vas, vascular lacuna of the nucleus of area postrema; Vd, dorsal nucleus of ventral telencephalic area; Vl, lateral nucleus of ventral telencephalic area; Vv, ventral nucleus of ventral telencephalic area; X, vagal nerve.

* Corresponding author.
E-mail address: ishwar@monash.edu (I.S. Parhar).

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In mammals, MT plays an important role in the homeostatic control of essential metals (copper and zinc), and protection of cells against heavy metal (cadmium and mercury) poisoning, intracellular oxidative damage, and ionizing radiation (Aschner et al., 1997; Boldrin et al., 2003; Knapen et al., 2005; Paul-Pont et al., 2012). Its synthesis is inducible by several factors including metals, inflammation, hormonal stimuli, glucocorticoids, endotoxin, cytokines, radiation, oxidative agents, stress and conditions that cause brain injury (West et al., 2008; Paul-Pont et al., 2012; Trinchella et al., 2008). Similarly in non-mammalian vertebrates, MT has been shown to be involved in heavy metal metabolism (Hamilton and Mehrle, 1986; Roesijadi, 1992). In the zebrafish, two *mt* genes exhibit different sensitivity towards exposition to metals and stress: *mt2* is sensitive to cadmium and cold shock treatment in zebrafish larvae, while *smtb* expression did not change in either treatment (Wu et al., 2008).

Despite the extensive studies on the role of MTs in heavy metal metabolism in the liver, the primary physiological role of MTs in the central nervous system (CNS) remains unclear. Moreover, studies of MTs in the brain of teleosts are limited to embryonic stage of zebrafish (Chen et al., 2004). The localization of MT isoforms in the brain might provide us valuable information regarding its function in the CNS. Thus in the present study, we localized the expression of *mt* mRNAs in the brain of adult zebrafish by *in situ* hybridization (ISH) to predict the potential role of MT isoforms in the brain of zebrafish. The *mt*-expressing cell types were characterized by double labelling combining ISH and immunofluorescence using antibodies to specific marker proteins for neurons (HuC/D) and astrocytes (glial fibrillary acidic protein [GFAP] and S100 protein). In addition, an antibody to proliferating cells nuclear antigen (PCNA) was used to explore if MT-expressing cells are proliferating.

2. Materials and methods

2.1. Animals

Adult (6–8 months) zebrafish were purchased from a local supplier and housed in 20 L fresh water aquaria, at 27 ± 0.5 °C with a controlled normal photo-regimen (14 h light–10 h dark). Fish were fed with adult zebrafish food (Zeigler, Gardners, PA) twice a day. Experiments were conducted after 2 weeks of acclimatizing to our laboratory conditions. All experimental procedures were conducted under ethical approval by Monash University Animal Ethics Committee (approval number: MARP/2012/094).

2.2. Comparative genomics analysis of MT genes

Sequences of zebrafish MT genes [*mt1*, *mt2* and *smtb*, GenBank/EMBL/DDBJ accession nos.: X97278.1 (also registered as NM_131075), NM_001131053.2 and NM_001201469.1, respectively] were retrieved from the National Center for Biotechnology Information (NCBI) database. Chromosomal synteny analysis was performed to determine gene orthology relationships. Chromosomal locations of MT genes in the human, mouse, zebrafish and Japanese medaka genome were identified using the UCSC Genome Browser (http://www.genome.ucsc.edu/) and Ensembl Genome Browser (http://www.ensembl.org/).

2.3. Tissue distribution of MT mRNAs in the zebrafish

Fish (n = 10; 5 male, 5 female) were anesthetised by immersion in 0.01% tricaine methanesulfonate (MS222, Sigma–Aldrich, St. Louis, MO) before the dissection of the tissue. Total RNA was isolated from the eye, brain, gills, kidney, heart, liver, ovary and skeletal muscle of female fish and from the testis of male fish with TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. RNA samples were treated with DNaseI (Promega, Madison, WI) to degrade DNA contamination in the samples. First strand cDNA was synthesized from 500 ng of the total RNA by using MultiScribe Reverse Transcriptase (Applied Biosystems, Foster City, CA) and 10× random primers (Applied Biosystems) in a 20 μL reaction mixture.

The cDNA were subjected to quantitative real-time PCR for *mt1*, *mt2* and *smtb* using ABIPRISM 7500 Sequence Detection System (Applied Biosystems). To normalize the level of target mRNA, *β-actin* mRNA (GenBank accession no. NM_131031) was quantified in the tissue cDNAs. The 10 μL reaction mixture contained 1× POWER SYBR Green PCR Master Mix (Applied Biosystems), 0.1 μM forward and reverse primers each (Table 1) and 1 μL of tissue cDNA. dH_2O was applied as a non-template control. The PCR reaction was carried out at 95 °C for 10 min and 40 cycles of 95 °C for 15 sec and 60 °C for 1 min. The PCR products were electrophoresed on 2.5% agarose gels, stained with ethidium bromide, and visualized by illumination under UV light. The nucleotide sequences of the PCR products were confirmed by sequencing.

2.4. *In situ* hybridization for MT genes

The sense and antisense digoxigenin (DIG)-labelled riboprobes for *mt2* and *smtb* were synthesized by *in vitro* transcription from the pGEM-T Easy vector (Promega, Madison, WI) containing 250- and 227-base pair (bp) fragments of zebrafish *mt2* and *smtb* cDNA, representing positions 78–327 bp and 21–247 bp, respectively (primer sequences are listed in Table 1). DIG labelling was achieved using MAXIscript (Ambion, Austin, TX) and DIG RNA labelling mix (Roche Diagnostics, Mannheim, Germany).

Zebrafish brains (male and female, n = 10) were fixed in 4% paraformaldehyde/phosphate buffered fixative for 6 h at 4 °C, cryoprotected in 20% sucrose solution, and embedded in Tissue Tek OCT compound (Sakura Finetechnical, Tokyo, Japan). Sagittal (n = 4) and coronal (n = 6) sections (14 μm thickness) were cut with a cryostat and thaw-mounted onto 3-aminopropylsilane (APS)-coated glass slides. DIG-*in situ* hybridization was performed as described previously (Ogawa et al., 2012). Briefly, sections were permeabilized with 0.2 M HCl for 10 min followed by proteinase K (1 μg/ml) treatment for 15 min, prehybridized at 58 °C for 2 h, and hybridized with DIG-labelled riboprobes (0.5 μg/ml) at 55 °C overnight in a humidified chamber. After hybridization, sections were washed and blocked with 2% normal sheep serum for 30 min at room temperature. DIG signals were detected with an alkaline phosphatase-conjugated anti-DIG antibody (Roche Diagnostics; diluted 1:500) and chromogenic development was achieved with 4-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate (Roche Diagnostics). The sections that had been stained were scanned, and the images were captured by using a Zeiss MIRAX Midi Slide scanning system (Zeiss, Gottingen, Germany) at a resolution of 230 nm using a 20X objective and processed with the Mirax Viewer Image Software (3DTech, Budapest, Hungary).

2.5. Double-labelling ISH with immunofluorescence

Cells expressing *mt2* and *smtb* mRNA were characterized by double-labelling with either a marker protein for neurons (HuC/D), astrocytes (GFAP and S100 protein) or proliferating cells (proliferating cell nuclear antigen, PCNA). ISH of *mt2* and *smtb* was carried out as described above. DIG signals were detected using Tyramide Signal Amplification (TSA) Plus Fluorescein kit (Perkin-Elmer, Wellesley, MA). After hybridization and the blocking step, color development reaction was

Table 1
Gene specific primers for real-time PCR and riboprobe synthesize.

Primer		Sequence (5′ → 3′)	Product size	Accession number
mt1 real-time	Forward	CTCTGGCTGCGTGTGTAAGG	108 bp	X97278.1
	Reverse	GCCCAGCACAGACGAACAA		
mt1 ISH	Forward	TGCCACCTGCAAGTGCACCAA	204 bp	X97278.1
	Reverse	AGCGAAGACGCCCAGCACAG		
mt2 real-time	Forward	TGTGGATACTCTCTGGAAAAATGG	185 bp	NM_001131053.2
	Reverse	AGCCACAGGAATTGCCTTTG		
mt2 ISH	Forward	GGACCCCTGCGAATGTGCCA	250 bp	NM_001131053.2
	Reverse	TGCGATGCAAAACGCAGACGTG		
smtb real-time	Forward	CTCATGCGCACACGACAAG	150 bp	NM_001201469.1
	Reverse	TGGTGTCACAGCGCATTACA		
smtb ISH	Forward	ACGCACGACGGAGCGATTCC	227 bp	NM_001201469.1
	Reverse	ACGCGCTCTCGCAGTCCTTC		
β-actin real-time	Forward	AGAGCTATGAGCTGCCTGACG	106 bp	NM_131031.1
	Reverse	CCGCAAGATTCCATACCCA		

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