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Molecular cloning and characterization of the expression pattern of the zebrafish α2, 8-sialyltransferases (ST8Sia) in the developing nervous system

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Abstract Sialyltransferases are Golgi type II transmembrane glycoproteins involved in the biosynthesis of sialylated glycolipids and glycoproteins. These sialylated compounds play fundamental roles in the development of a variety of tissues including the nervous system. In this study, we have molecularly cloned from zebrafish sources, the orthologues of the six human α2,8-sialyltransferases (ST8Sia), a family of sialyltransferases implicated in the α2-8-mono-, oligo-, and poly-sialylation of glycoproteins and gangliosides and we have analysed their expression pattern in the embryonic zebrafish nervous system, using *in situ* hybridization. Our results show that all six ST8Sia exhibit distinct and overlapping patterns of expression in the developing zebrafish central nervous system with spatial and temporal regulation of the expression of these genes, which suggests a role for the α2-8-sialylated compounds in the developing nervous system.

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Abbreviations

ST8Sia	α2,8-sialyltransferase, nomenclature according to Tsuji, S., Datta, A.K., Paulson, J.C., Glycobiology 6, v–vii (1996), Gangliosides nomenclature according to Svennerholm, L., J. Lipid Res. 5, 145–155 (1964)
ORF	open reading frame
hpf	hours post fertilization
dpf	days post fertilization
diSia	diSialic acid
Dre	Danio rerio
Hsa	Homo sapiens
ISH	in situ hybridization
NS	nervous system
CNS	central nervous system
PNS	peripheral nervous system

Introduction

Sialic acids (Sia) are a family of nine-carbon carboxylated monosaccharides, usually found at the non-reducing terminal position of sugar chains of glycoproteins and glycolipids (reviewed in [1]). Sialic acid residues are linked to either the 3- or 6-hydroxyl groups of galactose (Gal) or to the 6-hydroxyl group of *N*-acetylgalactosamine (GalNAc) or *N*-acetylglucosamine (GlcNAc). They may also form homopolymers of α2-8-linked residues, which are named according to the degree of polymerization (DP), di-(DP=2), oligo-(2<DP<7) or poly-sialic acid (DP>7) chains [2].

Decades of research have uncovered essential roles for both α2-8-sialylated glycolipids and glycoproteins in the central nervous system (CNS) [3]. Moreover, several severe diseases are characterized by abnormalities in either the biosynthesis or catabolism of these cellular components [4–6]. However, only a rudimentary understanding of the basic biological roles of these α2-8-sialylated compounds in the development of the CNS are known.

Gangliosides are a heterogeneous group of sialylated glycosphingolipids present in the outer leaflet of cell membranes, which contain highly antigenic carbohydrate moieties facilitating the production of antibodies and immunolocalization studies in the developing CNS [7]. DiSialylated gangliosides are known to play a role in neurite outgrowth and cell adhesion [8], differentiation and signal transduction. Although there are large differences between animal species and tissues, an increase in the content of gangliosides and changes in their degree of sialylation are observed along with the development of mammalian embryonic brain [9]. A shift from the simplest gangliosides of the a- and b-series (G_{M3} and G_{D3}) in mid-embryonic brains to more complex gangliosides such as G_{D1a}, G_{T1b} or polysialogangliosides of the c-series at later developmental stages has been reported [9, 10] for mouse and rat. Mass spectrometric analysis of zebrafish glycolipids extracted at different embryonic stages have shown that the major sialylated glycolipids contained up to four polymerized sialic acid residues [11]. Furthermore, cichlid fish brain was shown to be enriched with c-series gangliosides including G_{T3} and polysialo-species, suggesting a tissue-specific regulation of the gangliosides biosynthesis in brain [12].

Polysialic acid chains have been described in only a few animal brain glycoproteins [13–18]. In the developing nervous system (NS), it is well documented that polysialic acid chains (PSA) on N-CAM play fundamental roles in neural plasticity affecting axonal growth and fasciculation, cell migration, synaptic plasticity and neurogenesis (see reviews [19, 20]). In the NS of the developing zebrafish, PSA is transiently formed on N-CAM, where it acts in guiding outgrowing axons [21]. Furthermore, in mature brain, PSA N-CAM has been implicated in regulating adult neurogenesis [22] and in adult zebrafish brain it regulates cerebellar neuronal plasticity [23]. Di- and oligo-sialic acid containing glycoproteins have been known for a while [24] and Sato *et al.* have demonstrated that α2-8-linked diSialic acid (diSia) motifs occur in several glycoproteins of mammalian brain [14] among which CD-166, which appears to be involved in neurite formation in the Neuro2A mouse neuroblastoma cell line [25]. Occurrence of diSia motifs has been reported on N-CAM in the developing chick brain raising the question of its biosynthesis and function during vertebrate brain development [26].

The α2,8-sialyltransferases (ST8Sia), a subset of the animal sialyltransferases (Cazy GT-family # 29 [27]), catalyze the transfer of one to several sialic acid residues onto another sialic acid residue found on glycolipids or glycoproteins. In mammals, ST8Sia I and ST8Sia V are implicated in the biosynthesis of gangliosides, ST8Sia I (G_{D3} synthase) presenting a strict specificity towards G_{M3} resulting in the formation of G_{D3} and ST8Sia V (G_{T3} synthase) sialylating different gangliosides such as G_{D3}, but also G_{M1b}, G_{D1a} and G_{T1b} (reviewed in [28, 29]). The human ST8Sia VI catalyzes the transfer of a single sialic acid residue on sialylated *O*-glycans of glycoproteins leading to the formation of diSia motifs [30]. ST8Sia III catalyzes the transfer of one to several sialic acid residues either on glycoproteins or glycolipids [10] and is thought to be implicated in the biosynthesis of G_{T3} [10] and diSia motif of CD-166 [25]. Finally, poly-α2,8-sialyltransferases refer to the vertebrate ST8Sia II and ST8Sia IV. Both enzymes are expressed in the NS of most vertebrates where they catalyze the transfer of hundreds of sialic acid residues mainly on the *N*-glycans of N-CAM (reviewed in [31]) resulting in an increased neuronal plasticity and migration in embryonic vertebrate embryos (reviewed in [32]). ST8Sia II expression seems to be restricted to the early development stages among vertebrates, while ST8Sia IV has a more extended expression, from later development stages to adulthood in mammals, but at low level whatever the stage in zebrafish [23, 33].

Danio rerio serves as an excellent animal model for investigating the pattern of expression of ST8Sia genes since the embryos are optically transparent and readily accessible throughout early development stages. After the identification of all the human ST8Sia orthologues in the zebrafish genome [34], we report here on the molecular cloning and the expression pattern of the zebrafish ST8Sias as a first step to gain access to the implication of α2-8-sialylation of glycoconjugates during brain development in vertebrates.

Material and methods

Animals and maintenance

Zebrafish (*Danio rerio*) were maintained in our fish facility as previously described [35]. All experimental procedures adhered to the CNRS guidelines for animals in research.

RNA preparation and PCR detection of zebrafish ST8Sia

Danio rerio ST8Sia sequences identified *in silico* were PCR amplified with specific primers designed in the open reading frame to check expression and correct any

Table 1 Primer nucleotide sequences used for amplification of full-length open reading frame of zebrafish ST8Sias

	Human				Zebrafish			
ST8Sia	GB Acc. #	Chromosome	Primer	Sequence	cDNA source	Product (bp)	GB Acc. #	Chromosome
ST8Sia I	L32867, D26360, X77922	12p12	Forward	5′-TTTCTTGCAAT ACATCGGCGG	Kidney	1,095	AJ715535	4
			Reverse	5′-GTATTCTCATGC CTGTTGCAG				
ST8Sia II	U91641	15q26	Forward	5′-TTGCCCTG CGTTAGGAACCA	Kidney	1,271	AY055462	18
			Reverse	5′-TGTGTGTGTA AGGTCCAGTG				
ST8Sia III	AF004668	18q21	Forward	5′-CTGAAAGGAT GCGGGTTTCC	36 h embryos	1,252	AJ715543	24
			Reverse	5′-CATTAGTGCA ACCGAGACTC				
ST8Sia IV	L41680	5q21	Forward	5′-AGATGAGATGG GTGGTTATG	Brain	1,209	AJ715545	10
			Reverse	5′-ACAATGCCA AATTGGTCGAC				
ST8Sia V	U91641	18q12	Forward	5′-CCCGTGAGA ACGGTAAACAC	Brain	1,214	AJ715546[a], AM287263, AM287264	21
			Reverse	5′-TAAAGCACCT GCGACTAGCG				
ST8Sia VI	AJ621583	10p12	Forward	5′-AGAGCGGCA GCAGCATCTG	Kidney	1,183	AJ715551	3
			Reverse	5′-CATTTCCCACC AGCCTCGT				
ST8Sia VIIA	Not identified	Not identified	Forward	5′-TTTCCTGGTG GTCCTGAT	Ovary	1,101	AM287257	23
			Reverse	5′-CCTGTAATGTG CTCTCACATG			AM287258	23

Specific ST8Sia primer sets used for RT-PCR amplification of the full-length ORF of each zebrafish ST8Sia from various cDNA sources. Human ST8Sia cDNA and their zebrafish counterparts are indicated with corresponding GenBank accession numbers (GB Acc. #). In zebrafish, ST8Sia VII gene is duplicated on chromosome 23 (ST8Sia VIIA and ST8Sia VIIB) and ST8Sia VIIB is not transcribed in the zebrafish tissues studied

[a] The three accession numbers of zebrafish ST8Sia V correspond to splice variants of the transcript in the region encoding the stem region of the protein

sequencing error. A kidney cDNA library was kindly provided by Dr. L.I. Zon (Howard Hughes Medical Institute, Harvard Medical School, Boston, MA, USA). Total RNA was extracted from various zebrafish tissues: 36 hours post fertilization (hpf) embryos, adult brain and ovaries, using the Qiagen RNeasy kit, and quantified by spectrophotometry using the NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The integrity and purity of RNA preparations were analyzed by means of gel electrophoresis on Experion bioanalyzer (Experion, Bio-Rad Laboratories, Inc, Marnes-la-Coquette, France). For subsequent PCR amplifications, first-strand cDNA was synthesized from total RNA using the First Strand cDNA Synthesis kit according to the manufacturer's protocol in the presence of oligodeoxythymidilic acid$_{12–18}$. A specific fragment of about 300 bp was obtained after RT-PCR of RNAs isolated from various zebrafish tissues (data not shown). The Dre β-actin gene was amplified as a control of cDNA synthesis and purity. The RT-PCR products were subjected to 2% agarose gel electrophoresis.

Molecular cloning of full-length zebrafish ST8Sia

The zebrafish ST8Sia full-length open reading frame were cloned using a PCR based protocol from kidney cDNA library (ST8Sia I, ST8Sia II, ST8Sia VI), from total RNA isolated from 36 hpf embryos (ST8Sia III) or from adult brain (ST8Sia IV and ST8Sia V) (Table 1). Based on the gene nucleotide sequences determined *in silico*, primers (Eurogentec, Herstal, Belgium) were designed to the 5′- and 3′-UTR of each ST8Sia gene (Table 1). Additional primers within the previously described open reading frame (data not shown) were necessary to amplify overlapping regions within the coding sequences of ST8Sia IV and ST8Sia V and the resulting overlapping PCR products were subsequently annealed. PCR amplifications were carried out with hot-star Qiagen Taq polymerase (Qiagen, Courtaboeuf, France) using the buffer solution provided by the manufacturer. Annealing temperatures varied from 50 to 52°C. Full-length sequences were subcloned in the pCR$^{(R)}$2.1-TOPO vector (TOPO TA Cloning, Invitrogen, Cergy Pontoise, France) and nucleotide sequence was confirmed by sequencing (Genoscreen, Lille, France).

RNA probes

The cDNA fragments were then cloned in pGEM-T vector (Promega, Charbonnières-les-Bains, France). Linear templates were generated from this construct by performing a PCR amplification of the cDNA using the following primers: 5′-GGATCC**ATTAACCCTCACTAAAG**G GAATTTAGGTGACACTATA (that contains a T3 RNA polymerase promoter) and 5′-TAATACGACTCACTA TAGGG. After purification these templates have been used to generate the RNA probes. Both antisense- and sense-digoxigenin-labeled RNA probes were obtained using T7 or T3 RNA polymerase. After synthesis, RNA probes were purified with an RNA purification kit (Macherey-Nagel, Hoerdt, France) and checked for purity by denaturing agarose gel electrophoresis. Synthesized control sense probes gave no staining after whole-mount *in situ* hybridization (ISH).

Whole mount mRNA *in situ* hybridization

In situ hybridization was performed as described [36–38]. Embryos from AB/TU fish (a strain generated from crosses of two wild-type lines, AB and TU) are collected, dechorionated by pronase treatment, allowed to develop at 28.5°C until the appropriate stage and then fixed by incubation over night in 4% paraformaldehyde at 4°C. Embryos older than 24 hpf are incubated in 0.3× Danieau medium supplemented with 1-phenyl-2-thiourea (PTU, 0.003%) to prevent accumulation of pigment. After fixation, embryos are dehydrated and stored at −20°C in 100% methanol prior to ISH. The labeling reaction is monitored under a dissecting microscope and the reaction is stopped with 1× PBS at pH 5.5. Embryos are then mounted under a coverslip in 100% glycerol and incubated at least 24 h in the dark at room temperature prior to observation. Pictures are taken using a color CCD camera (Roper Scientific, Coolsnap) mounted on a dissecting microscope (Leica, M420) or on a compound microscope (Leica, DM RA2HC or Nikon, FXA).

Results

Molecular cloning, sequencing and expression

In order to identify the α2,8-sialyltransferase (ST8Sia) genes in the zebrafish genome, *Danio rerio* EST and genomic sequence databases were queried by BLAST search for sequences showing similarities to human ST8Sia genes [34]. The zebrafish ST8Sia genes were identified as single gene orthologues (Fig. 1a), with the exception of two ST8Sia related genes found tandemly duplicated on chromosome 23 in zebrafish genome that we named ST8Sia VIIA and ST8Sia VIIB (Table 1). All the identified zebrafish ST8Sia sequences retained the same genomic organization predicted for human genes [34] and the deduced protein sequences contained the sialylmotifs and family-motifs characteristic of the sialyltransferases and α2,8-sialyltransferases, respectively (Fig. 1b). Primer pairs were designed in the 5′- and 3′-UTR of each sequence in

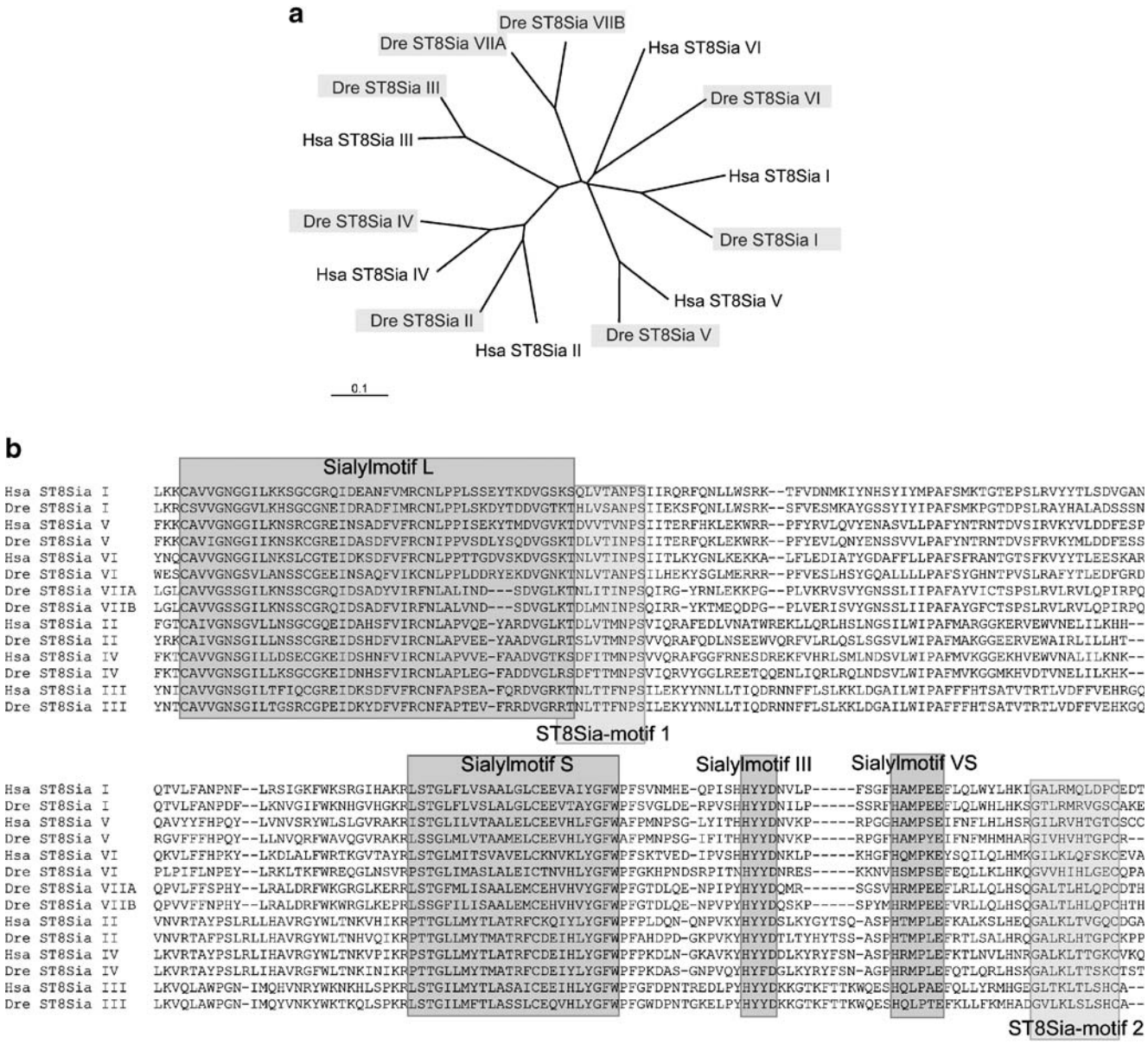


Fig. 1 **a** Unrooted phylogenetic analysis of zebrafish (*Dre*) and human (*Hsa*) ST8Sia sequences. Zebrafish and human ST8Sia protein sequences were aligned with clustal W at the PBIL. The aligned sequences were used for the construction of a tree using the maximum parcimony method with mega 3.1. This unrooted tree indicates the relationship of the zebrafish ST8Sia sequences to their human counterparts. **b** Comparison of the deduced amino acid sequences of the zebrafish and human ST8Sia catalytic domain. The sequences were aligned with Clustal W (PBIL) from the third amino acid residue upstream the sialylmotif L to the third amino acid residue downstream the ST8Sia-motif 2. The sialylmotifs L, S, III and VS above sequences are shaded in dark grey. The ST8Sia-family specific motifs 1 and 2 [59] below sequences are represented in light grey boxes. The last one is the C-term motif recently described (Harduin-Lepers *et al.* 2008, submitted)

order to amplify the full-length ORF and we cloned each of the zebrafish ST8Sia transcripts from various zebrafish tissues using RT-PCR (Table 1). Amplification products were subcloned and sequenced confirming the sequences of the predicted transcript and protein [34]. Deduced protein sequence comparison indicates that ST8Sia III is highly conserved from zebrafish to human (78% amino acid identity), whereas ST8Sia VI is less conserved, the zebrafish and human sequences sharing only 37% identity (Table 2).

Spatio-temporal expression of zebrafish ST8Sia mRNA

In a previous study, we analyzed the expression of zebrafish ST8Sias at different early developmental stages using a real time PCR approach (Chang *et al.* 2008, this issue) and we

Table 2 Zebrafish α2,8-sialyltransferases

	ST8Sia	Structures synthesized	Human protein size (AA)	Zebrafish protein size (AA)	Protein sequence identity
Poly α2,8-sialyltransferase	ST8Sia II (STX)	(Neu5Acα2–8)$_n$Neu5Acα2–3/6Galβ1–4GlcNAc- [a]	375	381	62%
	ST8Sia IV (PST)	(Neu5Acα2–8)$_n$Neu5Acα2–3/6Galβ1–4GlcNAc- [a]	359	358	69%
Oligo α2,8-sialyltransferase	ST8Sia III	Neu5Acα2–8Neu5Acα2–3Galβ1–4GlcNAc-(Neu5Aca2–8)$_n$ Neu5Acα2–3Galβ1–4GlcNAc- [b]	380	374	78%
Mono α2,8-sialyltransferase	ST8Sia I (G_{D3} synthase)	Neu5Acα2–8Neu5Acα2–3Galβ1–4Glc-Cer	356	339	60%
	ST8Sia V(G_{T3} synthase)	G_{D3}, G_{T3}, G_{D1c}, G_{T1a}, G_{Q1b}	376	374	72%
	ST8Sia VI	Neu5Acα2–8Neu5Acα2–3Galβ1–3GalNAc-0-Ser/Thr Neu5Acα2–8Neu5Acα2–6GalNAc-0-Ser/Thr	398	358	37%
	ST8Sia VII	Unknown	Not identified	358	–

Alternative sialyltransferase names are indicated in brackets. The sialylated compound formed, diSialylated motif (DP=2), oligosialylated motif (2<DP<7) or polysialylated motif (DP>7) [2] correspond the biosynthetic activity of mono-, oligo- or poly-α2,8-sialyltransferases, respectively. ST8Sia VII has no orthologous counterpart in the human genome

[a] $n>7$

[b] $2<n<7$

found that all the human ST8Sia orthologues were zygotically expressed during zebrafish embryo development. Moreover, we showed that, except for the ST8Sia II gene, ST8Sia genes lack maternal expression in the embryos (no PCR amplification detected at 0 hpf) and start to be expressed approximately at the first stage of segmentation (10 hpf). In order to assess the topographical distribution of zebrafish ST8Sia transcript at early stages of the zebrafish development, we used specific anti-sense digoxigenin-labelled RNA probes with no homology to other zebrafish sialyltransferases and whole-mount *in situ* transcript hybridization [37, 38]. Negative controls using sense ST8Sia probes did not produce detectable signal (data not shown).

No expression of the zebrafish ST8Sia III gene was detected in embryo until the early pharyngula period (24 hpf), when we detected the ST8Sia III gene in the forebrain marginal zone (telencephalon and diencephalon), in olfactory placodes, in cranial ganglia and spinal cord neurons (Fig. 2A, E). At 36 hpf, the zebrafish ST8Sia III gene was expressed in the forebrain and midbrain marginal zone, in the epiphysis and ventral part of the hindbrain (Fig. 2B, F) and from 48 hpf to 5 days, it was found in the brain marginal zone. The zebrafish ST8Sia III mRNA was also localized in the retina of the developing eye at 48 hpf and this expression was restricted to the inner cell layer in the retina at the larval stage (5 dpf) (Fig. 2C, D, G, H). No expression of the zebrafish ST8Sia III gene could be detected in the trunk.

Expression of the zebrafish polysialyltransferases ST8Sia II and ST8Sia IV genes has been described [23, 33, 39]. In a previous study, Thisse *et al.* 2001 reported an early onset expression of the ST8Sia II gene at 10 hpf in the neural keel, which correspond to presumptive central nervous system (direct data submission in ZFIN [39]). In this study, we did not observe ST8Sia IV gene expression in embryos until 24 hpf (Fig. 2) and the expression level of ST8Sia IV gene was generally weaker in the CNS throughout early developmental stages when compared to the one of ST8Sia II, previously reported. We have also observed strong basal level of expression of ST8Sia IV gene in the differentiating zebrafish brain, in a nucleus in tegmentum and in the ventral part of the brain from 24 hpf to 36 hpf (Fig. 2I, J, L, M). At 48 hpf, ST8Sia IV gene is expressed all over the brain and cranial ganglia (Fig. 2K, N).

There was no detectable expression of the zygotic ST8Sia I, ST8Sia V and ST8Sia VI genes before the early somitogenesis stage (10 hpf). In addition, the zebrafish ST8Sia VII gene was not expressed during embryonic development and the ST8Sia VI gene had a dispersed pattern of expression, not restricted to the NS (data not shown). At 10 hpf, low levels of ST8Sia I mRNA were first evident in the forebrain (telencephalon and diencephalon), but not in the ventricular zone as well as in the anterior part of the spinal cord and in one presumptive

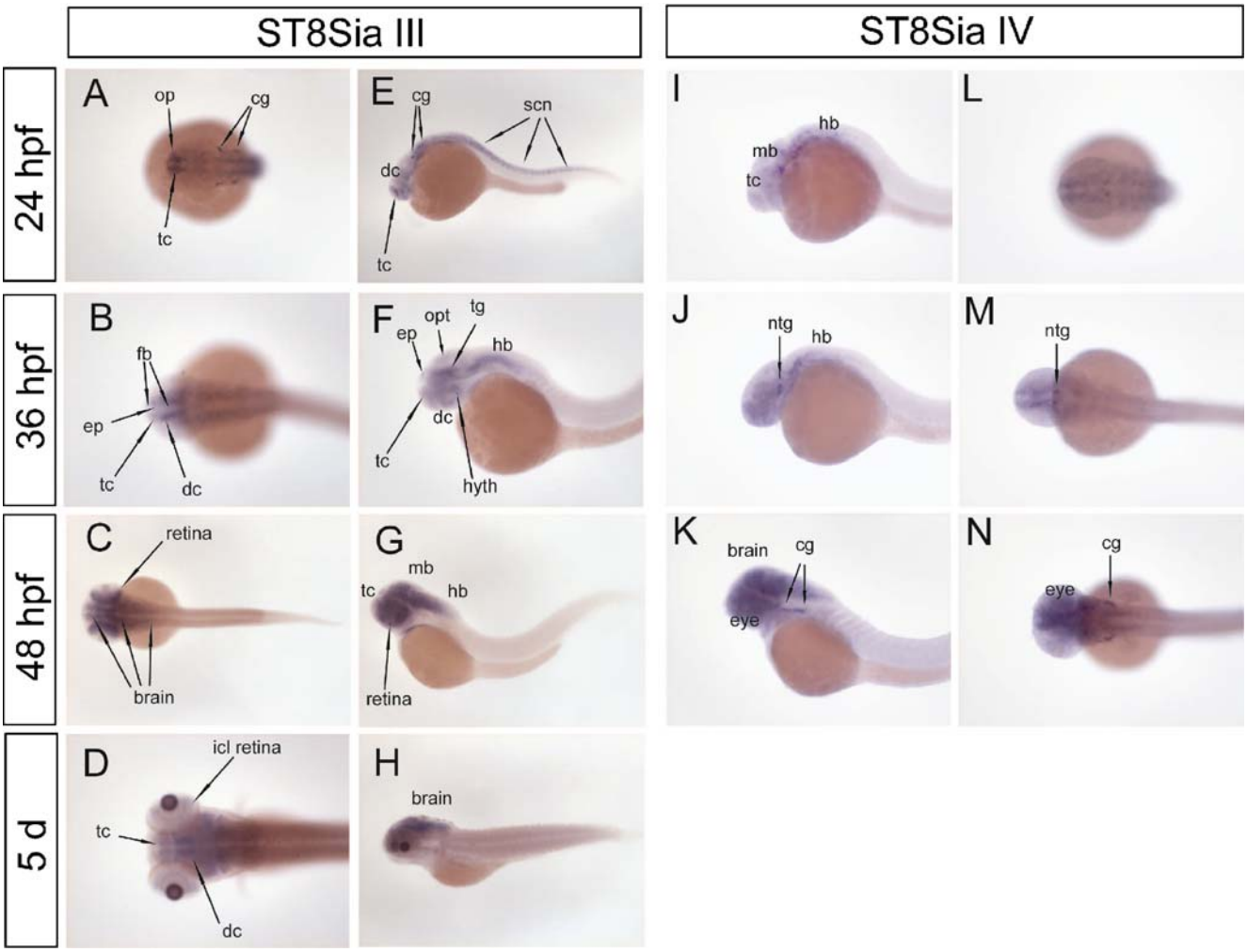
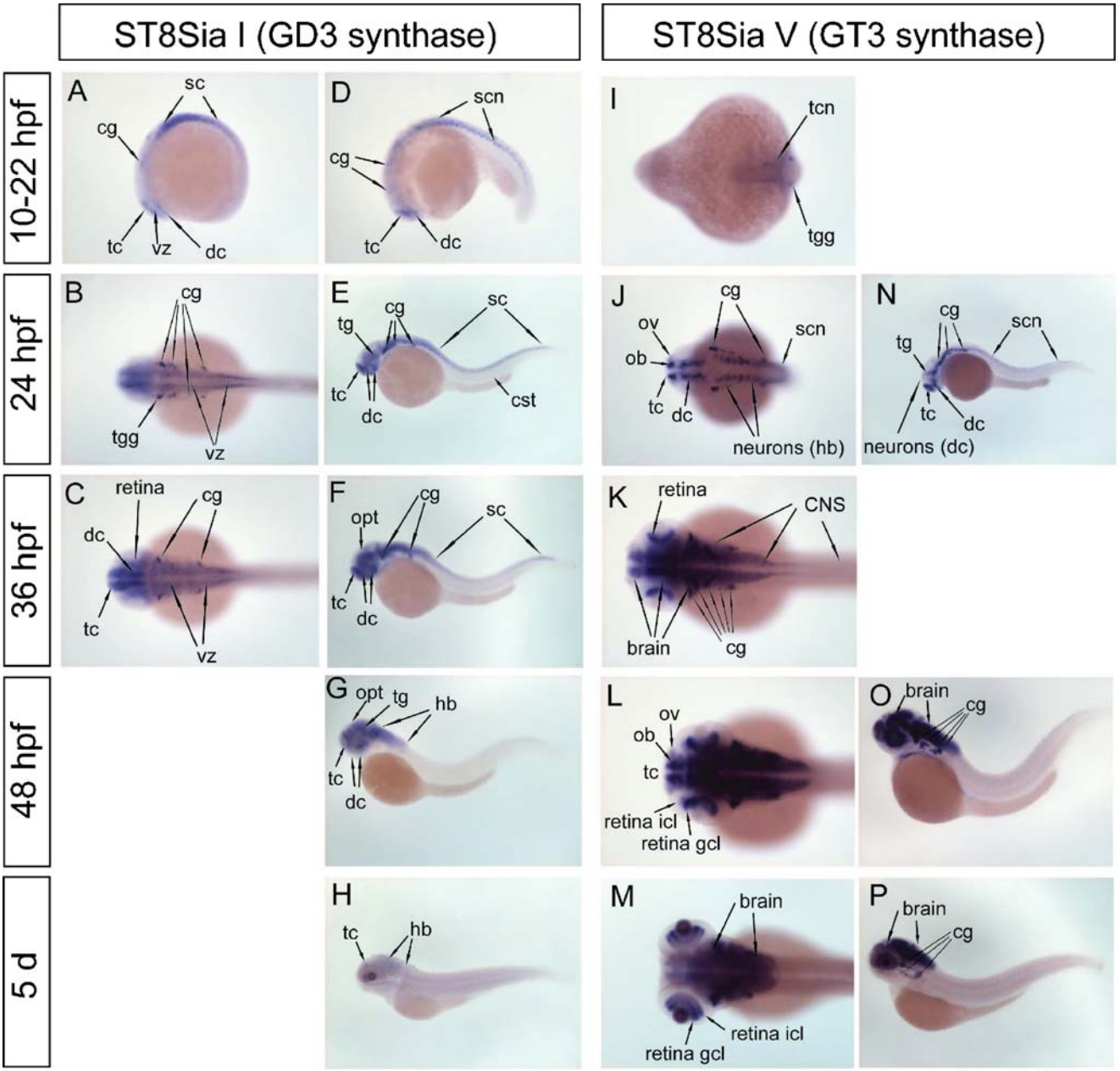


Fig. 2 Expression of the oligo-α2,8-sialyltransferases ST8Sia III gene and of the poly-α2,8-sialyltransferase ST8Sia IV gene in the differentiating central nervous system during embryonic and larval development of zebrafish. On the *left side* of the figure, panels **A**, **B**, **C**, **D** show dorsal and **E**, **F**, **G**, **H** show lateral views of the ST8Sia III gene expression (*anterior is to the left*). From pharyngula stage (24 hpf: **A**, **E**) to larval stage (5 dpf: **D**, **H**), expression of the ST8Sia III gene is mainly detected in forebrain marginal zone (telencephalon and diencephalon), in the olfactory placodes, cranial ganglia and spinal cord neurons (**A**, **E**). At 36 hpf, it is expressed in the epiphysis and ventral part of the hindbrain (**B**, **F**). At 48 hpf, expression of the zebrafish ST8Sia III is found in the retina and at 5 dpf, it is restricted to the inner cell layer in the retina. On the *right side* of the figure, panels **I**, **J**, **K** show lateral and **L**, **M**, **N** show dorsal views of the pattern of expression of the ST8Sia IV gene (*anterior is to the left*). From 24 hpf (**I**, **L**) to 36 hpf (**J**, **M**) strong basal level of expression of the ST8Sia IV gene is observed in the ventral part of the hindbrain and in a nucleus in tegmentum. At 48 hpf (K, N), ST8Sia IV gene is expressed in the whole brain and in cranial ganglia. *cg* Cranial ganglia, *dc* diencephalons, *ep* epiphysis, *fb* forebrain, *hb* hindbrain, *hyth* hypothalamus, *mb* midbrain, *ntg* nucleus in tegmentum, *op* olfactory placodes, *opt* optic tectum, *scn* spinal cord neurons, *tc* telencephalon, *tg* tegmentum

cranial ganglia (Fig. 3A). Later in somitogenesis (16 hpf), the ST8Sia I gene showed a similar pattern of expression in forebrain, in several cranial ganglia and in spinal cord neurons (Fig. 3D). By 24 hpf, the expression of the ST8Sia I mRNA extended to the whole CNS and shows a higher expression level in telencephalon with the exception of the ventricular zone (Fig. 3B), and a strong level of expression in the anterior and posterior lateral line of cranial ganglions (trigeminal ganglion, gV). Expression of the ST8Sia I gene was also detected in the midbrain (tegmentum) and in the ventral part of the hindbrain; no labelling was detected in optic tectum and weak labelling was observed in the corpuscle of Stannius, the fish organ responsible for the homeostasis of calcium (Fig. 3E). Peak expression of the ST8Sia I gene was reached by 36 hpf overall in the CNS and strong expression was detected in telencephalon, especially in olfactory bulb and in the peripheral nervous system (posterior and anterior lateral line ganglia). Weak expression was found in optic tectum and the corpuscle of Stannius was no longer detectable (Fig. 3C, F). At 48 hpf, the ST8Sia I gene was mainly detected in the brain excluded from the ventricular zone. A strong hybridization



was observed in the diencephalon subventricular zone as well as in ventral diencephalon, near the neurohypophysis (Fig. 3G) and in the hindbrain, two nuclei in rhombomere 3 expressed the ST8Sia I gene more strongly. A weak labelling was observed in the retina ganglion cell layer and the ST8Sia I gene expression was lost in cranial ganglia. At 5 dpf, expression of the ST8Sia I gene was weak and restricted to telencephalon and hindbrain (Fig. 3H). We did not observe ST8Sia V gene ISH in the developing zebrafish embryo until the middle of somitogenesis stage (22 hpf), when it was expressed in trigeminal ganglion and in telencephalon (Fig. 3I). By 24 hpf, the ST8Sia V mRNA was predominantly and highly expressed in olfactory vesicles, olfactory bulb, diencephalon marginal zone, in the lateral part of the hindbrain, tegmentum, cranial ganglia and in neurons of the dorsal part of the spinal cord (Fig. 3J, N). At 36 hpf, a high level of expression was detected in the CNS with the exception of the ventricular zone, in all cranial ganglia and in the ganglion cell layer of the retina (Fig. 3K). Levels of the ST8Sia V gene remained consistently high during pharyngula stage (24–36 hpf) and peak expression was reached by hatching period at 48 hpf (Fig. 3L, O). At that stage, we observed the same pattern of strong expression in the CNS and PNS. In addition, we

◀ **Fig. 3** Expression of the mono-α2,8-sialyltransferases ST8Sia I (GD3 synthase) and ST8Sia V (GT3 synthase) gene in the differentiating central nervous system during embryonic and larval development of zebrafish. Panels **B**, **C**, **I**, **J**, **K**, **L** and **M** show dorsal and **A**, **D**, **E**, **F**, **G**, **H**, **N**, **O**, **P** show lateral views (*anterior is to the left*). At early somitogenesis (10 hpf), ST8Sia I gene is expressed in the anterior part of spinal cord, in one presumptive cranial ganglion and in forebrain, but not in the ventricular zone (**A**) later on, at the middle of somitogenesis (18 hpf) it shows similar pattern of expression in forebrain, several cranial ganglia and spinal cord neurons (**D**). At 24 hpf, the staining denotes an ST8Sia I gene expression in the whole CNS, in midbrain and ventral part of hindbrain, stronger in telencephalon, with the exception of the ventricular zone. A strong expression is found also in cranial ganglia and a weak labelling in the corpuscles of Stannius (**B**, **E**). At 36 hpf, same observations are made and ST8Sia I gene expression is higher than at 24 hpf especially in telencephalon (olfactory bulb) and in the posterior and anterior lateral line ganglia. A weak ST8Sia I expression is detected in optic tectum and there is a small expression spot at the tip of the tail. The corpuscle of Stannius is no longer detectable (**C**, **H**). At 48 hpf, expression of the ST8Sia I gene is found in brain with the exception of the ventricular zone and strong expression in the diencephalon subventricular zone as well as in ventral diencephalon (near the neurohypophysis), no more expression was detected in cranial ganglia (**G**). At 5 dpf, expression of ST8Sia I gene is weak and restricted to the telencephalon and to the brain (**H**). Expression of the ST8Sia V gene starts in the middle of somitogenesis with strong labelling of trigeminal ganglion and telencephalon (**I**). At 24 hpf, expression of the ST8Sia V gene locates in the olfactory vesicle, olfactory bulb marginal zone of diencephalon, tegmentum, lateral part of hindbrain, in cranial ganglia and in neurons of the dorsal part of the spinal cord (**J**, **N**). A very strong level of expression in the CNS (non ventricular), in all cranial ganglia and in the ganglion cell layer of the retina appeared at 36 hpf (**K**) and at 48 hpf (**L**, **O**). In addition, there is a weak expression of the ST8Sia V gene in the inner cell layer and no expression in the proliferative zone (**L**, **O**). At 5 days, the ST8Sia V gene expression is very strong in brain (non ventricular zone) and in all cranial ganglia (**M**, **P**). *cg* cranial ganglia, *cst* corpuscle of Stannius, *dc* diencephalons, *ep* epiphysis, *fb* forebrain, *hb* hindbrain, *hyth* hypothalamus, *mb* midbrain, *ntg* nucleus in tegmentum, *op* olfactory placodes, *opt* optic tectum, *retina gcl* retina ganglion cell layer, *retina icl* retina inner cell layer, *sc* spinal cord, *scn* spinal cord neurons, *tc* telencephalon, *tg* tegmentum, *tgg* trigeminal ganglia, *vz* ventricular zone

detected a weak expression in the inner cell layer of retina for both ganglion and inner cell layer and no expression was found in the proliferative zone. At the larval stage (5 dpf), the level of expression of the ST8Sia V gene was still very high in the brain and in all cranial ganglia and no expression was detected in the ventricular zone of the brain (Fig. 3M, P).

Discussion

α2-8-sialylated glycoconjugates are ubiquitously expressed in many tissues of vertebrates and are differentially distributed in the NS. In particular, they are enriched in brain and spinal cord and they are known to undergo both qualitative and quantitative changes in sialic acid content during NS development [9, 32]. However, the regulatory mechanisms underlying these changes remain largely unknown. Among these mechanisms, which account for the variable expression of α2-8-sialylated glycoconjugates, there is the regulated expression the α2,8-sialyltransferases (ST8Sia) genes implicated in their biosynthesis. In a previous study, we identified several homologues of the known human ST8Sia in various vertebrate and invertebrate genomes that have evolutionary conserved genomic organizations and protein domain structures, and we found that the six orthologous ST8Sia genes are shared by zebrafish and mammals [34].

In this study we used the zebrafish as an animal model to analyze the spatio-temporal pattern of expression of the ST8Sia genes implicated in the biosynthesis of α2-8-sialylated glycoconjugates. The zebrafish is a simple model organism for assessing gene expression in the developing vertebrate NS [40] and offers a number of advantages over invertebrates such as *Drosophila melanogaster* or mammalian systems such as mouse. Zebrafish fertilization is external; the embryos develop rapidly *in vitro* and are transparent, which enabled us to visualize the early expression of the putative ST8Sia genes in the developing NS. Invertebrate models are also extremely powerful, but do not have α2-8-sialylated glycoconjugates, while higher vertebrate embryos have a greater complexity of NS and are not amenable to genetic analysis as zebrafish. The classical vertebrate model for genetic analysis has been mouse and single knock-out mice for ST8Sia I and for each of the two polysialyltransferases (ST8Sia II and ST8Sia IV) have been generated and characterized [32, 41–43]. Various phenotypes were observed corresponding to subtle changes in adult physiology. Double mutant mice for both ST8Sia II and ST8Sia IV resulted in complete loss of PSA in the perinatal brain and displayed severe defects in anatomical organization of the forebrain and wiring defects, progressive hydrocephalus, postnatal growth retardation and precocious death [32, 44, 45]. ST8Sia I gene knockout mice exhibited increased sensory responses to thermal and mechanical stimuli suggesting that the b- and c-series of gangliosides might be critical in the development and/or maintenance of the sensory NS [43]. Mice deficient in ST8Sia III, ST8Sia V and ST8Sia VI have not been created yet, but will provide insights into the role played by α2-8-mono- and oligo-sialylation during development, provided that these defects do not lead to embryonic lethality, because these stages of development are not easily accessible in mammals.

The oligo-α2,8-sialyltransferase ST8Sia III catalyzes the transfer of one to several sialic acid residues either on glycoproteins or glycolipids (Table 2) [28]. Even though its function remains largely unknown, it is thought to be implicated in the biosynthesis of the gangliosides G_{T3} [10] and the diSialylated motif of CD-166 [25]. Our ISH

experiment in the developing zebrafish embryo lacks expression of the zebrafish ST8Sia III gene until 24 hpf, which is in contrast to another recent study showing early expression of the ST8Sia III gene along the anterior–posterior axis of the developing embryo at the end of gastrulation (12 hpf) [46] and correlates with exact mRNA quantification by real time PCR data (Chang *et al.* 2008, this issue). Late induction of the ST8Sia III gene is also in agreement with the fact that oligosialylated glycolipids are exclusively detected in embryonic tissues from 24 hpf onwards (Chang *et al.* 2008, this issue). At 24 hpf we detected the ST8Sia III gene in the forebrain marginal zone (telencephalon and diencephalon), in olfactory placodes, in cranial ganglia and spinal cord neurons, at 36 hpf it is found in the forebrain and midbrain marginal zone, in the epiphysis and ventral part of the hindbrain and from 48 hpf to 5 dpf, it is found in the brain marginal zone (Fig. 2). Accordingly, structural analysis of glycolipids purified from these stages identified oligosialylated lactoceramides [11], some of which were described in adult cod brain [47]. As mentioned previously by Bentrop *et al*. [46], the ST8Sia III mRNA was also localized in the retina of the developing eye at 48 hpf and this expression is restricted to the inner cell layer of retina at the larval stage (5 dpf). Several sialylated glycoproteins such as PSA N-CAM or the sialoprotein associated with cones and rods (SPACR) expressed in the retina of fish [21, 23, 48], rodents [49, 50] or birds [51] might be the acceptor substrates for the ST8Sia III activity. Finally, no expression of the zebrafish ST8Sia III gene could be detected in the trunk, which is in contrast to the observation made by Bentrop *et al.* [46] of an intense expression of this gene between 20 hpf and 32 hpf in the fast muscle fibres of the entire myotome. These discrepancies might be explained by the use of different experimental setups.

Poly-α2,8-sialyltransferases refer to the two vertebrate sialyltransferases known as ST8Sia II (STX) and ST8Sia IV (PST), which are expressed in the CNS of vertebrates, where they catalyze the biosynthesis of PSA on N-CAM enabling the transfer of hundreds of sialic acid residues mainly on the *N*-glycans of N-CAM (Table 2) [28, 31]. This is related to their role in increasing neuronal plasticity and migration in embryonic vertebrates (reviewed in [32]. The ST8Sia II gene seems to have conserved the early development stage specific expression among vertebrates, while the ST8Sia IV gene has a more extended one, from later stages to adulthood in mammals, but at low level whatever the developmental stage in zebrafish [23, 33]. Expression of an orthologous ST8Sia II gene in zebrafish has been previously described in a high-throughput analysis (accessible on line, (ZFIN database at http://zfin.org) [39]) and thus ST8Sia II ISH was not repeated in these data set. It was shown that the onset of ST8Sia II gene expression occurred at about 10 hpf and remained relatively ubiquitous in the CNS until 48 hpf [39]. More recently, the molecular cloning and expression of the zebrafish ST8Sia II and ST8Sia IV homologues were reported by two other groups and both zebrafish polysialyltransferases were shown to be able to drive the expression of PSA on N-CAM [23, 33]. Marx *et al.* described very similar pattern of expression of the ST8Sia II gene in the central nervous system, forebrain, midbrain, neural tube and spinal cord at 22 hpf. Rieger *et al.* have studied the expression of ST8Sia II gene at later developmental stages and adult brain showing that, at 48 hpf, the ST8Sia II gene expression remains high along the dorsal hindbrain ventricle, in the differentiating cerebellum and in some cranial motor neurons, but was low in the telencephalon, diencephalon, tectum and anterior spinal cord. At 96 hpf, when migration ceases in the zebrafish NS, ST8Sia II gene expression levels declined in these regions and only faint expression remained in the cerebellum and along the ventricles. We did not observe ST8Sia IV gene expression in ovary (data not shown) and embryos until 24 hpf as previously described [23, 33], expression of this polysialyltransferase was generally weaker in the CNS throughout development when compared to ST8Sia II. Almost no expression of the ST8Sia IV gene could be detected at early embryonic stages by Marx *et al.* 2007 [33], whereas we could observe strong basal level of expression of ST8Sia IV gene in the differentiating zebrafish brain, in one nucleus in tegmentum and in the ventral part of the brain from 24 hpf to 36 hpf (Fig. 2), which is in agreement with the observation made by Rieger *et al.* 2008. These slight differences in ISH observations could simply result from different experimental setups. At 48 hpf, ST8Sia IV gene is expressed overall in brain and cranial ganglia (Fig. 2). Interestingly, no expression of the polysialyltransferase genes was detected in the developing nor in the adult zebrafish retina, even though PSA N-CAM was described in adult rodent retina [49, 50], in the retinotectal pathway of adult goldfish [52], and in the developing zebrafish retina [21, 23, 48] suggesting that another ST8Sia gene expressed might be implicated in the biosynthesis of PSA N-CAM in the retina.

Mammalian ST8Sia I, ST8Sia V and ST8Sia VI are considered as mono-α2,8-sialyltransferases catalyzing the transfer of a single sialic acid residue on another sialic acid residue (Table 2). The human ST8Sia VI catalyzes the transfer of single sialic acid residues on sialylated *O*-glycans of glycoproteins leading to the formation of diSia motifs on *O*-glycans and does not use glycolipids as acceptor substrates [30]. Our *in situ* analysis illustrates a dispersed pattern of expression of the ST8Sia VI gene, that is not restricted to the NS (data not shown), suggesting a potential implication of α2-8-sialylated *O*-glycosylproteins in brain development. ST8Sia I and ST8Sia V are known in mammals to be

implicated in the biosynthesis of gangliosides [28]. ST8Sia I (G_{D3} synthase) presents a strict specificity towards G_{M3} resulting in the formation of G_{D3}, whereas ST8Sia V (G_{T3} synthase) is able to sialylate different gangliosides such as G_{D3}, and also G_{M1b}, G_{D1a} and G_{T1b}. ST8Sia I, ST8Sia III and ST8Sia V genes were analyzed by RT-PCR using specific primer sets in developing mouse brain, and no significant difference in the expression pattern during mouse brain development was observed [9]. Our ISH experiment in the developing zebrafish embryo did not show any detectable expression of the zygotic ST8Sia I and ST8Sia V before the early somitogenesis stage (10 hpf), which is in accordance with the quantification of ST8Sia and ST8Sia V mRNAs by real time PCR (Chang *et al.* 2008, this issue). The ST8Sia I mRNA was detected in the CNS, mainly in the forebrain with the exception of the ventricular zone, in spinal cord and also in the cranial ganglia. The ST8Sia I gene expression in the NS peaks at 24 hpf and was restricted to the telencephalon and hindbrain at 5 dpf (Fig. 3). Similar pattern of expressions have been reported in the early developmental stages of mouse brain using northern blot, RT-PCR, and ISH [53, 54] and more recently, Luque *et al.* reported on a very similar temporal and spatial pattern of expression of the *Xenopus laevis* ST8Sia I gene [55]. In a previous study, Sohn *et al.* examined the expression of the zebrafish ST3Gal V (G_{M3} synthase) gene using whole mount ISH and showed an ubiquitous pattern of expression with a high level in the CNS. Overexpression of the ST3Gal V transcript in the developing zebrafish led to neuronal cell death particularly apparent in the forebrain, midbrain and mid/hindbrain boundary indicating that these brain regions are sensitive to G_{M3} over expression [56]. Overlapping expression pattern in the same brain regions of the ST3Gal V and ST8Sia I genes is also indicative of a regulated pathway of gangliosides use in brain. The ST8Sia V gene had a similar pattern of expression in the developing CNS and PNS, with unusual high level of expression in cranial ganglia. The peak expression was reached by hatching period at 48 hpf. In addition, high expression level of the ST8Sia V gene was reached at hatching (48 hpf) in the ganglion cell and inner nuclear layers of the neural retina. This overlapping expression pattern in the retinal neuroepithelial cells of the ST8Sia I, ST8Sia V and ST8Sia III genes implicated in the gangliosides biosynthesis is in good agreement with high ganglioside concentrations described in retina [57] and suggests an important role of gangliosides in retina development [58].

In this reverse genetic approach, we aimed to determine the spatio-temporal pattern of expression of each human ST8Sia genes orthologue during zebrafish embryonic development. Our studies with whole mount ISH during early zebrafish embryogenesis illustrate a predominant expression of the ST8Sia genes in the CNS with the notable exception of the ST8Sia VI gene, which exhibited an ubiquitous pattern of expression. In addition, we observed overlapping pattern of expression among ST8Sia genes mainly in the developing NS and retina suggestive of common functions of the ST8Sia genes and uncovering potential functions of α2-8-sialylated glycoconjugates during CNS and retina formation.

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