Characterization of the polysialylation machinery in fish

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List of Abbreviations

BLAST	Basic local alignment search tool
CAZy	Carbohydrate-active enzyme
CMAH	CMP-Neu5Ac hydrolase
C. maraena	*Coregonus maraena* – Maraena whitefish
CMAS	CMP-Sia-synthase
CMP	Cytidine monophosphate
CNS	Central neuronal system
CTP	Cytidine triphosphate
DA	Dopamine
DDX4	DEAD box helicase gene 4
DiSia	Disialic acid
DP	Degree of polymerization
FB	Fore brain
FSGD	Fish-specific genome duplication
EndoN	Endoneuraminidase
Gal	Galactose
GalNAc	N-Acetylgalactosamine
GlcNAc	N-Acetylglucosamine
GNE	UDP-GlcNAc-2 epimerase/ManNAc kinase
GT29	Glycotransferases family 29
Kdn	Deaminated neuraminic acid
ManNAc	N-Acetylmannosamine

List of Abbreviations

MonoSia	Mono sialic acid
MSA	Multiple sequence alignment
NA	Noradrenaline
NANP	N-Acylneuraminate-9-phosphatase
NANS	Neu5Ac-9-phosphate synthase
NCAM	Neural cell adhesion molecule
NCBI	National center for biotechnology information
Neu	Neuroaminic acid
Neu5Ac	N-acetylneuraminic acid
Neu5Gc	N-glycolylneuraminic
N-Term	N-terminal
OligoSia	Oligo sialic acid
PBR	Poly basic region
PDB	Protein data bank
PolySia	Poly sialic acid
PolyST	Polysialyltransferase
PSGP	Polysialoglycoprotein
PSTD	Polysialyltransferase domain
qRT-PCR	quantitative real- time- polymerase chain reaction
RNA	Ribonucleic acid
RND	Rounds of duplication
SaGD	Salmonidae genome duplication
S. lucioperca	*Sander lucioperca* – pikeperch
Sia	Sialic acid

List of Abbreviations

SM-L	Sialylmotif-large
SM-S	Sialylmotif-small
SM-VS	Sialylmotif-very small
ST	Sialyltransferase
ST3Gal	β-Galactoside α2,3-Sialyltransferase
ST6Gal	β-Galactoside α2,6-Sialyltransferase
ST6GalNAc	β-N-Acetylgalactosaminyl α2,6-Sialyltransferase
ST8Sia	α2,8-sialyltransferase
TGD	Teleost genome duplication
TM	Transmembrane domain
UDP	Uridine diphosphate
WB	Western blot
WGD	Whole genome duplication
5-HT	Serotonine

1. Introduction

1.1 Glycans

Glycans have an important biological role in single and multicellular organisms. Their chemistry and metabolism have been important topics since 1970. Without any known exceptions, a dense complex barrier of glycoconjugates surrounds all eukaryotic cells in nature; that group of sugar barrier is better known as glycocalyx (Varki et al. 2008). Due to numerous glycan acceptors on the membrane, the whole eukaryotic cell surface is coated by a thick layer of proteoglycans, glycoproteins, and glycosphingolipids (Lindhorst 2000; Varki 2008, 2017). Recently, it was discovered that small RNAs can also serve as a scaffold for glycosylation. The glycoRNAs decorate the surface of the cells as well (Flynn et al. 2021). All these glycoconjugates are involved in many biological processes (Cummings and Pierce 2014) and serve in several crucial processes as mediators for ligand-receptors and cell-cell interactions within cell communication and cell differentiation, as well as within immunological and neurological events (Green et al. 1995; Crocker, Paulson, and Varki 2007; Varki 2017; Kelm and Schauer 1997; Schauer 2000; Ohtsubo and Marth 2006; Varki and Angata 2006; Varki 2007; Varki and Varki 2007; Sato 2004).

In vertebrates, sialic acids (Sias) are one of the most frequent terminal residues of glycans. Due to its negative charge and the outermost position, this sugar modulates numerous different important mechanisms, such as cell differentiation, cell growth, cell signalling, and fertility (Varki and Schauer 2009).

1.2 Sialic acids (Sias)

Sias are 9-carbon backbone monosaccharides, hosting a carboxyl group at C-1 and a keto group at C-2 (Schauer 2004; Angata and Varki 2002; Schauer 1996). The basic structure is neuraminic acid (5-amino-3,5-dideoxy-D-glycerol-D-galacto-non-2-

ulosonic acid [Neu]), which has an amino group at C-5 and does not exist in this form in any other known structure (**Figure 1**). The most common and well described derivates are obtained by modification at the C-5 position: N-Acetylneuraminic acid (Neu5Ac), N-Glycolylneuraminic acid (Neu5Gc), and deaminated neuraminic acid (Kdn) (**Figure 1**) (Galuska et al. 2010).

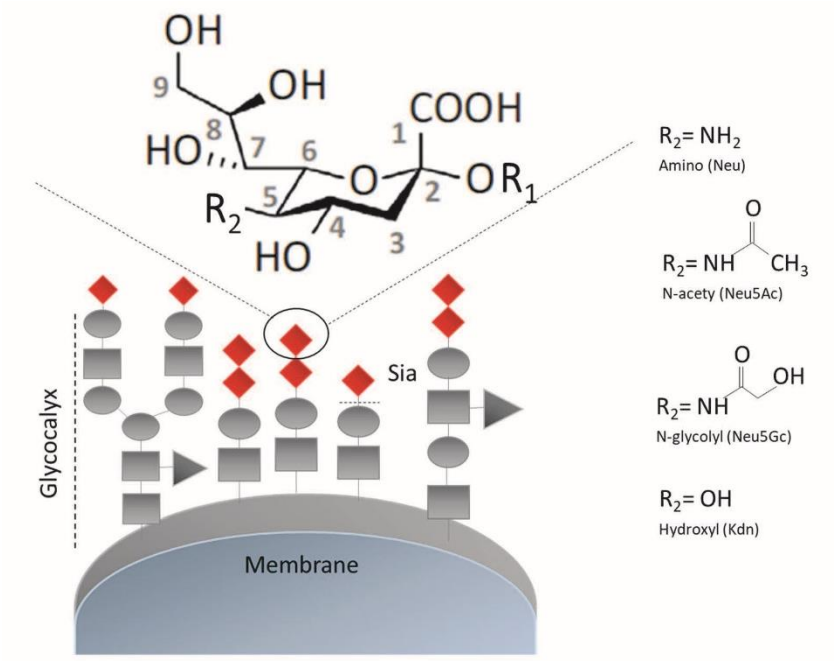

Figure 1: Schematic representation of Sias on the cell surface

The structure of Sias consists of a nine carbon backbone and a modification on Carbon 5 with an amino (Neu), a N-acetyl (Neu5Ac), a N-glycolyl (Neu5Gc) or a hydroxyl group (Kdn). The symbol for Sias, the red diamond, is based on the symbol nomenclature for glycans (https://www.ncbi.nlm.nih.gov/glycans/snfg.html). Inspired from (Tiralongo and Martinez-Duncker 2013; Büll et al. 2016).

The high diversity of the three forms of Sias is caused by chemical modifications of one or more hydroxyl groups located at the positions of C-4, C-7, C-8 or C-9 (**Figure 2A**). Possible substituents are acetyl, sulfonyl, lactyl, methyl, or lactone residue, which results in a modified Sia form (acetylation, sulfation, lactylation, and methylation) (Angata and Varki 2002; Schauer 2004, 2009) (**Figure 2B**).

The most common Sia found in nature is Neu5Ac, which is present in all vertebrates (Varki 2009). Neu5Gc has been found in mammals, as well as in fish and in a few birds and reptiles (Schauer et al. 2009). The presence of Kdn is uncommon in amphibian; however, it has been found in fish as organ-specific sialylation patterns with Kdn being found essentially in the intestine (Yamakawa et al. 2018) (Inoue and Kitajima 2006). More rarely is the occurrence of Kdn in mammals; only traces of Kdn have been detected in mammals´ malignant tumours (Devine et al. 1991) (**Figure 2E**). Interestingly, Sias can be linked to each other by α2,4-, α2,5$O_{glycolyl}$-, α2,8-, α2,9-, or α2,8/9-linkages (Sato and Kitajima 1999; Miyata, Sato, and Kitajima 2007) (**Figure 2C**). However, in mammals, only polymers of α2,8-linked-Neu5Ac residues were described, whereas in fish eggs, a high variety of different polySia polymers were observed (Inoue and Iwasaki 1978; S. Inoue et al. 1987). Homopolymers as well as hybrid structures of these sialic acid species can be present in fish eggs (Sato et al. 1993). Sugar polymers consisting of Sia residues can exhibit a high variation in the degree of polymerization (DP), which is between 2 and 400 residues (**Figure 2D**) (Sato and Kitajima 2013). Thus, Sias can form mono, di-, oligo-, and polymer structures: mono sialic acids (monoSia, DP 1), disialic acids (diSia, DP 2), oligomers (oligoSia, DP 3-7), and polysialic acids (polySia, DP ≥8) (Sato 2004; Sato et al. 1998; Sato and Kitajima 2013) (**Figure 2D**).

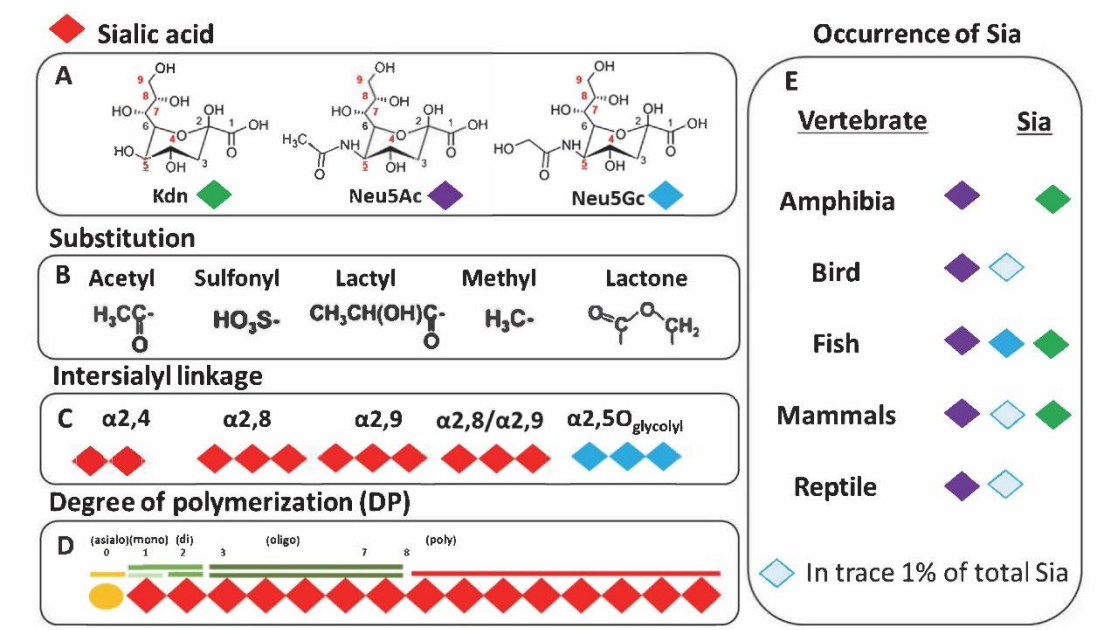

Figure 2: Structures and characteristics of Sia.

A: Chemical structure of the three major Sias: Kdn, Neu5Ac and Neu5Gc. **B**: Basic structure of potential substituents: acetyl, sulfonyl, lactyl, methyl and lactone group. **C**: Intersialyl linkages of polymerized Sia: α2,8-, α2,9-, α2,8/9, α2,4-, and α2,5-$O_{glycolyl}$-linkages. **D**: the degree of polymerization (DP) of polymerized Sia structure: mono: DP = 1; di: DP = 2; oligo: DP = 3–7 and poly: DP = 8 up to 400). **E**: summarized occurrence of Sia in vertebrate phyla. Red (Sia) and green (Knd), violet (Neu5Ac) and blue (Neu5Gc) diamonds are based on the symbol nomenclature for Glycans (https://www.ncbi.nlm.nih.gov/glycans/snfg.html). Figure based on (Tiralongo and Martinez-Duncker 2013; Schnaar, Gerardy-Schahn, and Hildebrandt 2014; Sato and Kitajima 2020)

1.3 Sialylation of Glycans

1.3.1 Synthesis of Sias

The starting product for Sia synthesis is UDP-N-acetylglucosamine (UDP-GlcNAc) received from glycolysis. Within the cytosol, three enzymes are involved in a four-step process. A bi-functional enzyme, UDP-GlcNAc-2 epimerase/ManNAc kinase (GNE), catalyses the two initial steps. In the first step, UDP-GlcNAc is converted to N-Acetylmannosamine (ManNAc) via the epimerase function, then ManNAc is further phosphorylated to ManNAc-6-P by the kinase activity. ManNAc-6-P is the

precursor for the following Sia biosynthesis of Neu5Ac. Following, Neu5Ac is formed by the condensation and dephosphorylation reactions, which are catalysed by Neu5Ac-9-phosphate synthase, also called sialic acid synthase (NANS) and Neu5Ac-9-phosphate phosphatase or N-acylneuraminate-9-phosphatase (NANP), respectively (Li and Chen 2012; Varki and Schauer 2009). Neu5Ac can be converted to Neu5Gc by CMP-Neu5Ac hydrolase (CMAH). The enzymatic activity of CMAH was lost during the human evolution because of a genetic deletion (Varki 2001). Thus, there is no production of Neu5Gc. The broadly expression of Neu5Gc in human may help to evade some pathogens, because it seems to be that pathogens prefer to bind Neu5Gc as NeuAc (Varki 2001, 2009). In the nucleus, Sia is converted to the activated form, CMP-sialic acid, using CTP as a donor, enabling the transfer of Sia to further glycans. Therefore, the activated sugar donors return to the cytosol and are subsequently transferred into the Golgi apparatus by a nucleotide sugar transporter, where sialylation takes place (Varki and Schauer 2009). The elongation of glycans using Sias is catalysed by sialyltransferases (STs) (**Figure 3**).

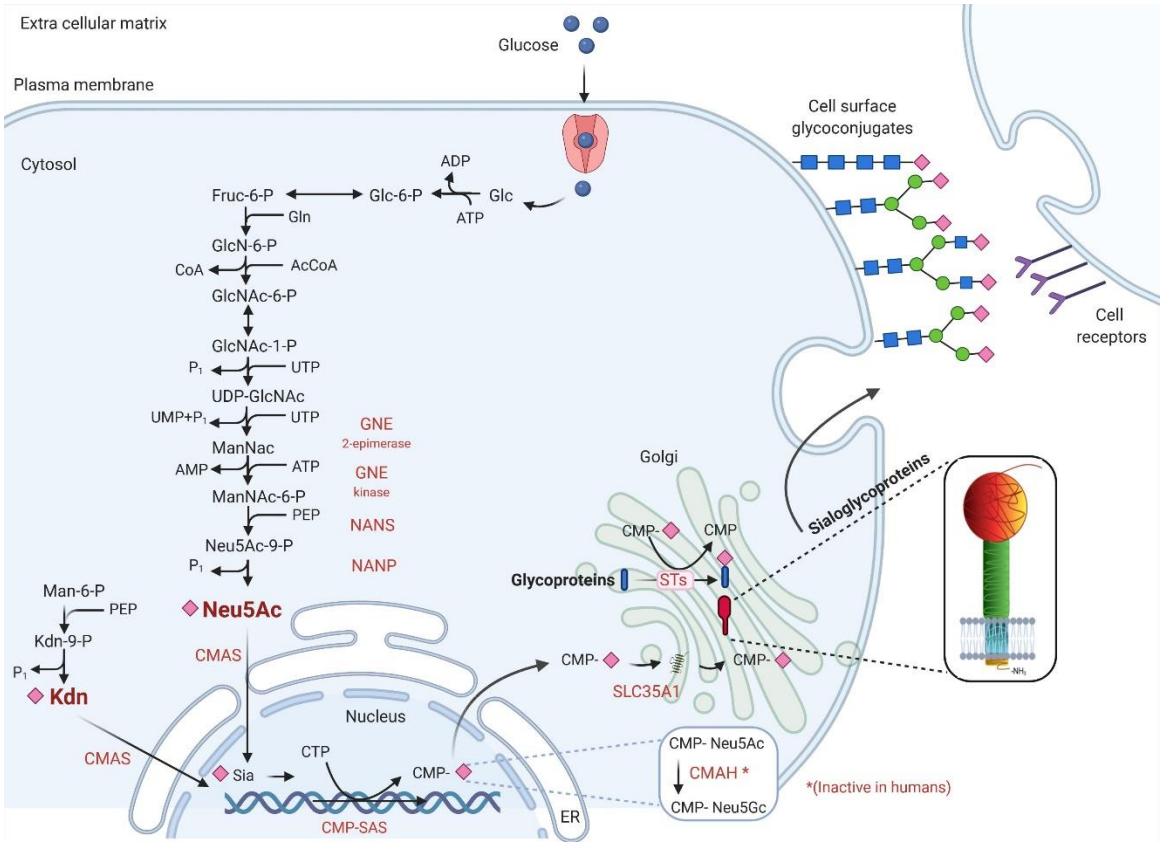

Figure 3: Schematic pathway of Sias biosynthesis in vertebrates.

The schematic illustration shows the pathway for the biosynthesis and activation of the most common Sias present in nature. The biosynthesis starts from glucose in the cytosol. GlcN-6P is converted into the sugar nucleotide UDP-GlcNAc, which is converted afterwards in ManNAc by a bifunctional enzyme, called GNE. In the first step UDP-GlcNAc is converted to ManNAc via the epimerase function, whereas ManNAc is further phosphorylated to ManNAc-6-P by the kinase activity. In the following sialic acid (Neu5Ac) is formed by the condensation and dephosphorylation reactions which is catalysed by NANS and NANP (Li and Chen 2012; Varki and Schauer 2009). For the production of Kdn instead, mannose-6-phospate is used. Both, Neu5Ac and Kdn, will be activated in the nucleus by CMAS. CMP-Neu5Ac can be converted in the cytoplasm to Neu5Gc by CMAH. This enzyme is inactive in humans (Varki 2001). CMP-Sias are the donor sugar-nucleotides for the sialylation process. Therefore, are transported into lumen of the Golgi apparatus by the transporter SLC35A1, where they are used by different STs to produce α2,3, α2,6 and α2,8 sialoglycoproteins. (Varki 2001) Figure based on (Petit et al. 2018; Schauer 2004). Figure created with BioRender.com.

1.3.2 Sialyltransferases (STs)

In vertebrates, the transfer of CMP-Sias to the terminal non reducing position of galactose (Gal), N-acetylglucosamine (GlcNAc), N-acetylgalactosamine (GalNAc) or Sia monosaccharides of glycoconjugates acceptors requires a subset of different STs, which catalyses the creation of α2-3-, α2-6-, and α2,8- glycosidic linkages.

All animal STs, identified up to now, are grouped in the glycotransferases family 29 (GT29) of the carbohydrate-active enzyme (CAZy) database of glycosyltransferases (Lombard et al. 2014) (Coutinho et al. 2003). They are characterized by their common organization (Lombard et al. 2014), the linkage (Harduin-Lepers et al. 2008; Harduin-Lepers 2010; Patel and Balaji 2006) and the origin (Lombard et al. 2014; Petit et al. 2018). The GT29 family, in turn, is sub-divided into four families of proteins:

1. **The β-Galactoside α2,3-Sialyltransferase family (ST3Gal)**, which catalyse the transfer of Sia in an α2,3-linkage to the terminal of β-galactopyranosyl (Gal) residues on glycoproteins and glycolipids (Harduin-Lepers et al. 2005; Harduin-Lepers 2013).

2. **The β-Galactoside α2,6-Sialyltransferase family (ST6Gal)**, which catalyse the transfer of Sia to the terminal of Gal residues of disaccharides through an α2,6-linkage on glycoproteins and glycolipids (Dall'Olio 2000).

3. **The β-N-Acetylgalactosaminyl α2,6-Sialyltransferase (ST6GalNAc) family**, which catalyses the transfer of Sia onto GalNAc residues (Ikehara et al. 1999).

4. **The α2,8-Sialyltransferase family (ST8Sia)**, which catalyses α2,8-linkages of one or several Sia residues to another Sia of glycoproteins or glycolipids (Harduin-Lepers et al. 2005; Harduin-Lepers 2013).

All these enzymes are involved in post-translational glycosylation of proteins in the Golgi apparatus of cells from vertebrates (**Figure 3)** (Paulson J.C and Colley 1989).

1.4 ST8Sia family

The six members of the mammalian ST8Sia family catalyse the transfer of Sias to sialic acid residues of glycoproteins or glycolipids by the formation of α2,8-linkages. Up to now, there have been six characterised ST8Sia sub-families in mammals, which can be divided into three further groups (**Figure 4**):

1. The group of mono-α2,8-sialyltransferases includes the sub-family of ST8Sia I, ST8Sia V, ST8Sia VI. They catalyse the transfer of a unique Sia residue via a α2,8-linkage (Harduin-Lepers 2010).
2. The second group of oligo-α2,8-sialyltransferases comprises the sub-family of ST8Sia III, which catalyses the transfer of one or more Sia residues either on glycoproteins or glycolipids and oligoSia can be formed (Yu et al. 1988) (Angata et al. 2000; Harduin-Lepers 2010).
3. The third group includes the two poly-α2,8-sialyltransferases (polysialyltransferases, polySTs) sub-families ST8Sia II and ST8Sia IV. Both enzymes were identified in bony fish and mammals as well as invertebrates. They can catalyse the formation of long polymers (Harduin-Lepers 2010).

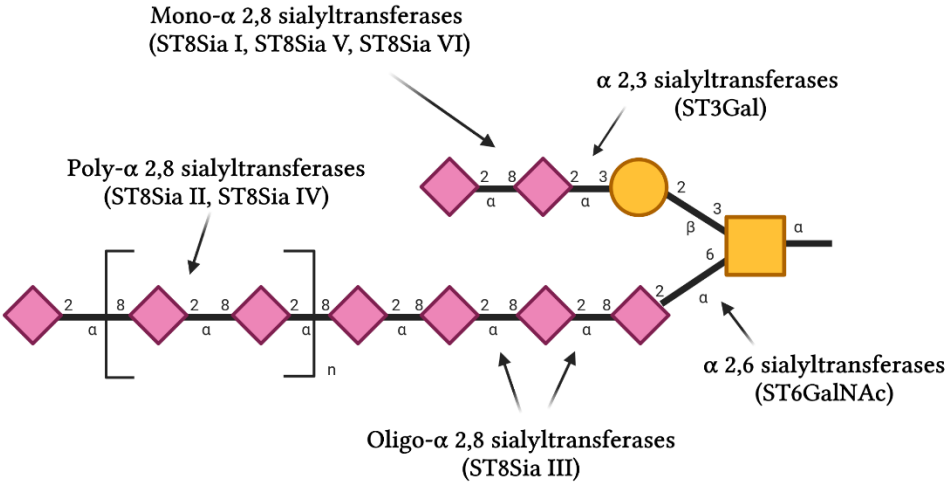

Figure 4: Schematic representation of the sialyltransferases families.

The schematic illustration shows the GT29 family: mono-α-2,3-STs (ST3Gal), mono-α2,6-STs (ST6GalNAc), and α2,8-STs (ST8Sia): mono-STs (ST8Sia I, V, VI, VII), oligo-STs (ST8Sia III), and poly-STs (ST8Sia II and IV).
The pink diamond indicates Sia, the yellow circle Gal and the yellow square GalNAc, the representation is based on the symbol nomenclature for Glycans (https://www.ncbi.nlm.nih.gov/glycans/snfg.html). Figure created with Biorender.com

1.4.1 Structural features of ST8Sia family

The GT29 family is sub-divided into four families, which are characterised by conserved regions called family-motifs. Sialylmotifs (SM) were identified in all STs in vertebrates and invertebrates and are specific for all enzymes of that family. Moreover the SM are important hallmarks used for the identification of the STs genes (Harduin-Lepers et al. 2001).

The STs are type II transmembrane glycoproteins (Paulson J.C and Colley 1989). They have a short N-terminal cytoplasmic tail, a transmembrane domain, a stem region, and a large C-terminal catalytic domain oriented toward the lumen of the Golgi apparatus (**Figure 5**). The cytoplasmic tail is usually short with a medium value of ten amino acids. Its amino acid sequence is highly variable and not very well conserved across animal evolution (Petit et al. 2010). The transmembrane

domain (TMD) consists of 16-20 amino acids and is highly variable in its composition. Moreover the TMD is rich in leucine (L) and phenylalanine (F) in the central part, which is consistent with the TMD being embedded in the hydrophobic milieu of the Golgi membrane (Patel and Balaji 2007). The stem region (SR) is located about 50 amino acids upstream of the catalytic domain and is not essential for its activity, even though its function is to extend the catalytic domain into the Golgi apparatus (Patel and Balaji 2007).

The catalytic domain consists of about 250 to 300 amino acids oriented in the lumen of the Golgi (Harduin-Lepers 2013). Four conserved motifs were identified in the catalytic domain using multiple sequencing alignment (MSA) (Datta 2009) they are implicated in the catalytic activity (Jeanneau et al. 2004).

1. The sialylmotif large (SM-L) contains 48–49 amino acids and 5 of them are strictly conserved across the evolution (Livingston and Paulson 1993). SM-L is implicated in the formation of disulphide bonds with the cystines in the sialylmotif small (Datta, Chammas, and Paulson 2001; Rao et al. 2009), that has been shown to be essential for polySTs activity (Patel and Balaji 2006) and in donor substrate binding (CMP-sialic acid) (Datta and Paulson 1995; Datta, Sinha, and Paulson 1998).

2. The sialylmotif small (SM-S) contains 23 amino acids (Datta and Paulson 1997) and is also implicated in the formation of disulphide bonds (Datta, Chammas, and Paulson 2001) (Rao et al. 2009). This motif is involved in the acceptor and donor substrate binding (Datta, Sinha, and Paulson 1998).

3. The sialylmotif III (SM-III) has four amino acids in which His (H) and Tyr (Y) are highly conserved (Jeanneau et al. 2004). Both amino acids are required for optimal activity and receptor recognition (Kapitonov and Yu 1999; Jeanneau et al. 2004).

4. The sialylmotif very small (SM-VS) has six amino acids and is highly conserved. In particular, His (H) has been conserved and is needed for the catalytic activity (Geremia, Harduin-Lepers, and Delannoy 1997; Kitazume-Kawaguchi, Kabata, and Arita 2001). Site- directed mutagenesis of H residues in ST8Sia II and ST8Sia IV did not induce any conformational modification nor bind substrate but did cause a loss of polySTs activity (Kitazume-Kawaguchi, Kabata, and Arita 2001).

The SMs are involved in the recognition of donor and acceptor substrates (Datta and Paulson 1995; Datta, Sinha, and Paulson 1998).

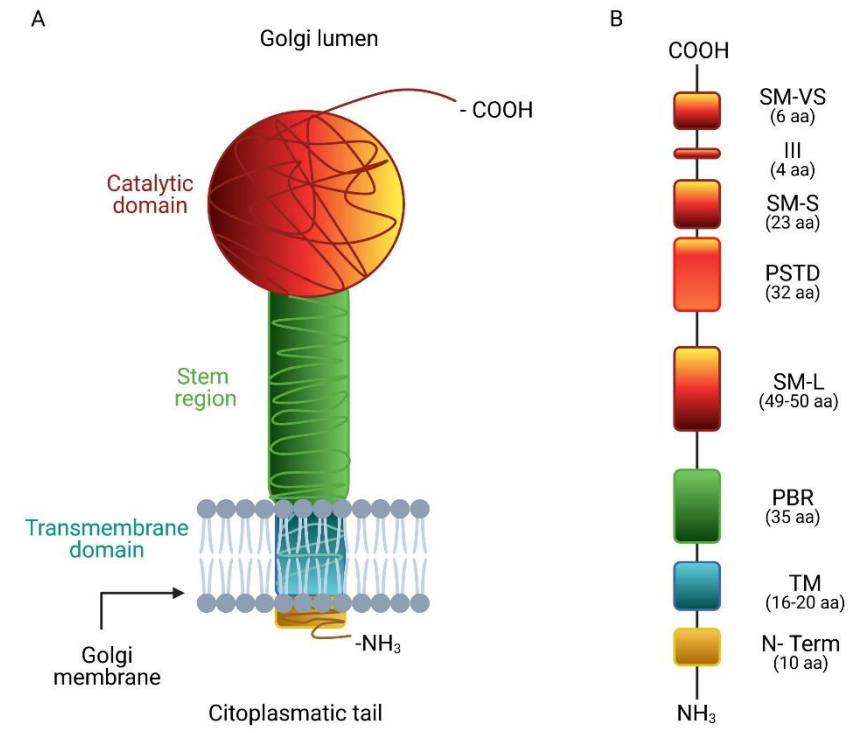

Figure 5: Topology and representation of glycosialyltransferase

A: Schematic representation of sialyltransferases consisting of a short N-terminal cytoplasmic tail (yellow), a transmembrane domain (blue), a stem region (green), and a catalytic domain (red). **B**: representation of the complete protein domain structure of polyST showed the different regions. TM (blue), PBR, and PSTD (red), SM-L SM-S, III, and

SM-VS (dark red). Inspired from (Patel and Balaji 2007; Datta 2009; Paulson J.C and Colley 1989; Bhide, Fernandes, and Colley 2016). Figure created with BioRender.com.

1.4.2 Polysialyltransferases (polySTs)

The human polySTs ST8Sia II and ST8Sia IV share about 60% sequence identities (Datta and Paulson 1995). The peculiarity in those enzymes are two additional sequence motifs called polysialyltransferase domain (PSTD) and polybasic region (PBR) (Foley, Swartzentruber, and Colley 2009; Nakata, Zhang, and Troy 2006) (**Figure 5**). The PSTD is a polybasic motif located upstream to the SM-S, which is involved in the interaction with polySia formed (Foley, Swartzentruber, and Colley 2009; Nakata, Zhang, and Troy 2006). It consists of a sequence of 32 amino acids located between the amino acids 246-277 in ST8Sia IV and 261-292 in ST8Sia II (Nakata, Zhang, and Troy 2006; Sevigny et al. 1998; Kitazume-Kawaguchi, Kabata, and Arita 2001). The PSTD domain is required for polysialylation (Foley, Swartzentruber, and Colley 2009) and the deletion of this region results in the loss of its activity (Hebert, Garman, and Molinari 2005). All positively charged amino acids are required for an optimal polysialylation of the neural cell adhesion molecule (NCAM), which is the main and best known polySia carrier in mammals (Nakata, Zhang, and Troy 2006).

The other unique polybasic motif is PBR with a sequence of 35 amino acids located in the stem region of the enzyme, which seems to be involved in the protein-specific polysialylation. It is located between the amino acids 71–105 in ST8Sia IV and between 80–120 amino acids in ST8Sia II (Foley, Swartzentruber, and Colley 2009). Site-specific mutations in that region decreases NCAM and autopolysialylation (Foley, Swartzentruber, and Colley 2009).

The polySTs are mainly expressed in the nervous system (NS) of most vertebrates (Kojima et al. 1996; Mühlenhoff et al. 1996). Further, they were discovered in different tetrapods and have been cloned from them (Nakayama et al. 1995;

Scheidegger et al. 1995). The latest investigations have shown, that the polySTs are also present in the teleost branches (Harduin-Lepers et al. 2005), and enzymatic assays show a very low enzymatic activity towards NCAM (Asahina et al. 2006).

1.5 The biological role of Sias and its polymers

1.5.1 The biological role of Sias

As previously mentioned, Sias play a special role in different physiological processes because they are located at the outermost position of glycans. For this reason, they are able to mediate cell-cell recognition and modulate different interactions of cells with toxins, hormones, or pathogens (Neu, Bauer, and Stehle 2011; Kurosawa et al. 1997) by distinct mechanisms (Varki and Schauer 2009). Furthermore, they can modulate the hydration of proteins, such as mucins, on the cell surface, because of their hydrophilic and negatively charged character (Varki and Gagneux 2012). In mammals, Sia residues are mainly recognized by selectins and sialic acid-binding immunoglobulin-like lectins (siglecs), which represent essential regulators of the immune system.

Remarkably, changes in the sialylation status of cells are frequently associated with several diseases. Some changes are involved in degenerative diseases, like atherosclerosis and diabetes, as well as neurological disorders, such as Alzheimer´s (Varki and Schauer 2009). The importance of Sia residues is underlined by the fact that an inactivation of the sialylation process leads to a lethal phenotype in mice (Schwarzkopf et al. 2002).

1.5.2 Sia polymers

In mammals, Sias are mainly present as monomers on glycans but also long polySia chains can be present (Schauer 2004). As previously mentioned above (section 1.4), biosynthesis of polySias in mammals, is carried out by the two related enzymes ST8Sia II and ST8Sia IV (Nakayama et al. 1995; Livingston and Paulson

1993; Petit et al. 2018; Harduin-Lepers et al. 2008).Interestingly, polySia has different physiological functions. For instance, the long negatively charged chains have anti-adhesive properties (Rutishauser 2008). This characteristic influences the cell-cell interactions negatively and increases the migration possibility of precursor cells (Yang, Major, and Rutishauser 1994), the major carrier in vertebrate brain is NCAM. Furthermore, a large amount of active molecules bind directly to polySias, like neurotransmitters (dopamine, norepinephrine, epinephrine) (Sato, Yamakawa, and Kitajima 2010), and neurotrophic factors, growth factors (Ono et al. 2012), brain-derived neurotrophic factor (BDNF), and vascular endothelial growth factor (VEGF) (Kanato, Kitajima, and Sato 2008; Strubl et al. 2018). Furthermore, these charged polymers are able to bind histones as well and influence histone-mediated cytotoxicity (Zlatina and Galuska 2019). In addition, polySia influences the activity of lactoferrin on the release of neutrophil extracellular traps (Kühnle et al. 2019).

The most studied polySia-carrier is NCAM, a member of the immunoglobulin (Ig) superfamily (Fux et al. 2003). NCAM is mainly expressed in brain tissues (Colley, Kitajima, and Sato 2014). During embryonic development, polysialylated NCAM is expressed in the whole brain and is involved in different events like cell migration and axonal outgrowth (Rønn, Berezin, and Bock 2000). Both polySTs, ST8Sia II and IV, are highly expressed in mouse brain (Nakayama et al. 1995; Livingston and Paulson 1993). However, *in vivo* studies showed that ST8Sia II is the predominant enzyme in mouse brain during the primary development (embryonic, perinatal, and early postnatal development), whereas ST8Sia IV is predominant in the adult brain (Ong et al. 1998). Experiments on ST8Sia II and ST8Sia IV knockout mice demonstrated that polySia plays an essential role in learning processes, cognitive flexibility, and adaptability, as well as in establishing fear memories (Markram, Gerardy-Schahn, and Sandi 2007). Remarkably, the

simultaneous knock out of both α2,8-polySTs leads to a lethal phenotype in mice demonstrating the importance of polySia for mammals (Weinhold et al. 2005). Interestingly, in mammals, only α2,8-linked polymers consisting of Neu5Ac could be detected, whereas several different polySia species like the O-acetylated polymers of Neu5Ac, Neu5Gc, and Kdn homopolymers, as well as hybrid structures of those Sia species could be present in fish eggs (Sato et al. 1993). The different forms of homo and heteropolymers include: poly(Neu5Ac), poly(Neu5Gc), poly(Neu5Gc), poly(Kdn) and hybrids poly(Neu5Ac, Neu5Gc) with acetylation on different carbon sites (C-4, C-7, C-9) (Sato et al. 1993). Other studies demonstrated that fertilized (Inoue and Iwasaki 1978) (Iwasaki and Inoue 1985) and unfertilized (S. Inoue et al. 1987) salmonid eggs contain different Sia polymers, in terms of type of Sia and type of linkage (Kanamori et al. 1990; Kitajima, Inoue, and Inoue 1986). In fish eggs, the main protein carriers of polySia are the polysialoglycoproteins (PSGPs), whereas NCAM is more expressed in vertebrate embryonic brain (Iwasaki and Inoue 1985; Kitajima et al. 1984). In contrast to NCAM, where polySia are on N-glycans, PSGPs contain polySia on O-glycans (Kitajima et al. 1984). PSGPs are a unique class of glycoproteins, which are related with the exocytosis of vesicles named cortical alveoli (Inoue and Inoue 1986). Those are founded on the peripheral cytoplasm of Salmonidae fish eggs (Kitajima, Inoue, and Inoue 1986).

However, the occurrence of Sia polymers during oogenesis and its other functions in fish have not been well known . For this reason, we wanted to analyse the distribution of polySTs, the polysialylation status in ovaries, and its possible role during stress situations in teleost.

1.6 Teleostei

1.6.1 Teleost evolution

Vertebrate lineage went through several genome doubling rounds, named whole genome duplication (WGD). In particular, two events or rounds of duplication (RND): [RND1 ~550 million years ago (mya) and RND2 ~500 mya] happened at the root of early vertebrate evolution before the divergence of ray-finned and lobe-finned fish (Dehal and Boore 2005) (**Figure 6**). WGD-spurred innovation was especially relevant in the case of teleost fish, which colonized a wide diversity of habitat on Earth, including many extreme environments between 552 and 474 mya. With the WGD began the major radiation of about 30,000 fish species on our planet (Amores et al. 1998). Teleost evolution started more than 400 mya after the two earlier rounds of WGD (Betancur et al. 2013). 225-333 mya ago (Amores et al. 1998), a third fish-specific genome duplication (FSGD- RND3), also called teleost-specific duplication (TGD), occurred (Santini et al. 2009; Meyer and Van de Peer 2005). TGD was responsible for the diversification of the major extant fish lineage, ray-finned-fish (Actinopterygii), which constitutes approximately half of the vertebrates on the Earth (Nelson 2006). After the TGD, additional genetic events occurred in different teleost lineages (Uyeno and Smith 1972; Larhammar and Risinger 1994). In particular, a forth additional round of genome duplication, the salmonid-specific duplication (SaGD- R4) (Macqueen and Johnston 2014; Berthelot et al. 2014), took place 25 to 100 mya at the base of the salmonids branch (Allendorf and Thorgaard 1984).

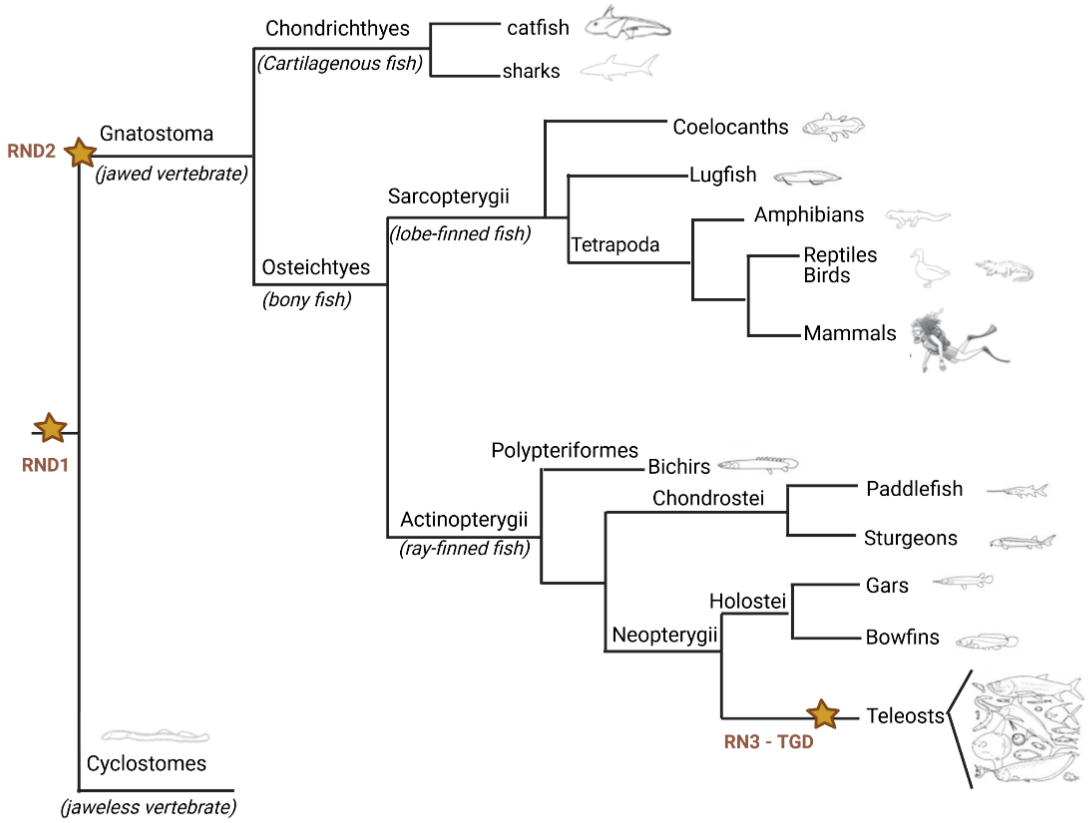

Figure 6: Phylogenetic tree of vertebrate

Schematic phylogenetic tree of vertebrate evolution. Two rounds of genome duplication (RND1 and RND2 yellow stars) occurred before the Gnatostoma divergence. The gnatostoma branch is divided into two categories: Chondrychtyes (cartilagenous fish, shark, and rays) and Osteichthyes (bony fish). The teleost evolution started after the bony fish radiation and divided into two categories: Sarcopteryggi (lobe-finned fish that contain Tetrapods) and Actinopterygii (ray-finned fish which contain Neopterygii inclusive Chondrostei, Holeostei and Teleostei). The Actinopterygii members include Teleosts, when the TGD occurred (RND3 yellow star). Figure inspired from (Yamamoto, Bloch, and Vernier 2017; Suzuki, Brandley, and Tokita 2010). Figure created with BioRender.com.

After such genetic events, the destiny of the duplicated genes in a genome can be different. During a duplication event, the duplicated genes can be lost or maintained in a genome. The gene loss played essential roles in speciation (Lynch and Conery 2000). However, gene duplication also triggered specializations. The genes can specialize by partitioning ancestral gene functions on duplicated genes (subfunctionalization) or by acquisition of new functions on the new duplicated

gene (neofunctionalization) (Force et al. 1999; Teppa et al. 2016) (Figure 7). These events generated several homologous genes from the same ancestral gene (paralogues in the same species and orthologues in different species), and it is therefore complicated to understand the function of each newly described gene. In addition, the presence of an additional gene on the chromosome does not necessarily mean that the gene has a special role or is functionally active. Due to the fourth important genetic event, the salmonid family became an important animal model to explore the impact of polyploidization in teleosts in comparison to the other teleost that did not undergo additional duplication.

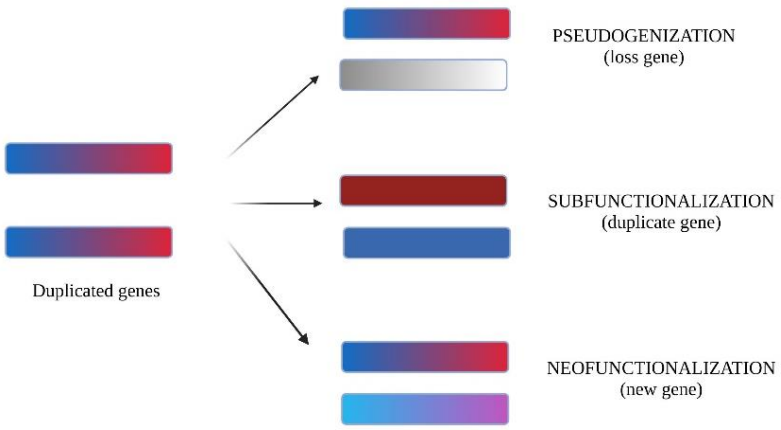

Figure 7: Evolutionary fate of genes after WGD

Schematic representation of the fate of duplicated genes. On the left site, two duplicated genes (blue/red coloured boxes). On the right site, the three destiny of the genes after the WGD, pseidogenization, subfunctionalization, neofunctionalization. Figure inspired from (Teppa et al. 2016). Figure created with Biorende.com.

1.6.2 Baltic Sea fish

The Baltic Sea fish are commercially and culturally important species because of environmental characteristics. They can live in brackish water environments or in fresh-marine water. The slightly salty water conditions result in an increased growth rate for many fish. Moreover, they are promising candidates for freshwater aquaculture diversification. Additionally, they belong to a category of high-quality

food with high nutritional and notable economic value, while also having soft and tasty flesh. Two of the most used aquaculture Baltic Sea species in the last years are the Maraena whitefish and the pikeperch.

The maraena whitefish (*Coregonus maraena* [*C. maraena*], *Linneus* 1758) is part of the anadromous and landlocked population inserted in different parts of North Europe, like Germany, Poland, and Scandinavia (Kottelat 1997; Kottelat 2007). This population is typically born in freshwater, foraging along coasts toward adulthood, and spawning in freshened parts of estuaries or in lower rivers (Kottelat 2007). Since middle 1990, efforts started to cultivate them in a semi-intensive pond recirculating aquaculture system (RAS) (Bochert, Horn, and Luft 2017). The taxonomic classification of *C. maraena* is complicated because it is part of the family of Salmonidae and belongs to the sub-family of Coregoninae (**Figure 8**). Those family genes undertook a supplementary duplication of the genome (SaGD-R4) with a consecutive variation around 95 mya (Macqueen and Johnston 2014; Berthelot et al. 2014).

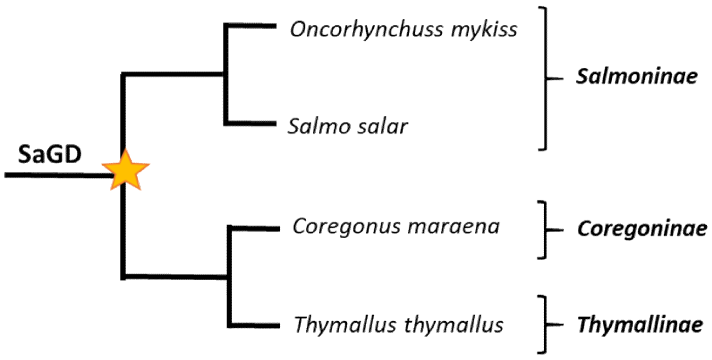

Figure 8: Salmonidae taxonomy

Representation of the phylogenetic relation of Salmonidae family. *Oncorhynchus mykiss*, *Salmo salar* (Salmoninae), *Coregonus maraena* (Coregoninae), and *Thymallus thymallus* (Thymallinae). Salmonidae genome duplication (SaGD yellow star). Figure based on (Macqueen and Johnston 2014).

Another important fish group of freshwater natives of the northern hemisphere is the Percidae family. This family contains more than 11 genera and more than 200 species. Its morphologic characters suggest that this family diverged 66–58 mya (Stepien and Haponski 2015) (**Figure 9**).

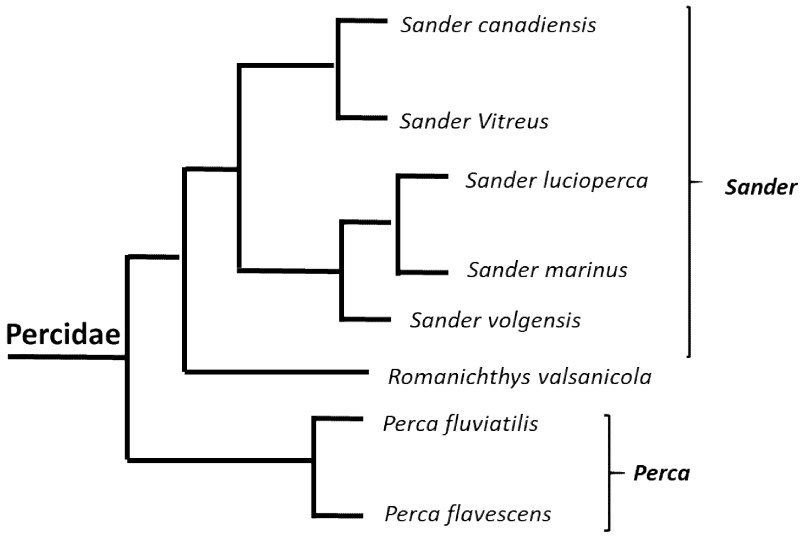

Figure 9: Percidae taxonomy

Representation of the phylogenetic relation of percidae family. Figure based from (Haponski and Stepien 2013).

The pikeperch (*Sander lucioperca [S. lucioperca], Linneus. 1758*) is the most common fish from the Percidae family. It is a fresh and brackish water fish (Kestemont, Dabrowski, and Summerfelt 2015), found in water bodies with low salinities from Germany, like the river Elbe, up to China, passing through the Baltic and the Caspian Seas (Freyhof and Kottelat 2008). Like Coregonous, it is an important anadromous high economical fish food, more often used in the last ten years for rearing in aquacultures to take advantage of its fast-growing potential in commercial production. It is a valuable recreational and commercial fish species (Rundberg 1977), for which reason it has been actively stocked and spread by man as well as loaded into lakes.

1.7 Fish ovary

The specific peculiarities of the reproduction system in every species are an adaptation to the particular conditions of reproduction and development, which provide the essential replenishment for the preservation of the species' survival (Vlaming 1972). The fish follow different strategies and tactics to produce their progeny (Wootton 1990), which depends on the species and the environment. Fish vary in terms of diversity of reproductive strategies. They can be gonochoristic (sex fixed after maturation) or hermaphroditic (sex may change after maturation) (Murua and Saborido-Rey 2003), and they can spawn one to multiple times during the season (Wootton 1990). Most Teleostei are dioecious or gonochoristic, which means that they have separate sex organs. The male bears testicles producing spermatozoa, while the female carries ovaries, where the oocytes are produced (Patino and Redding 2000). Those fish can have two types of gonadal development. Therefore, the ovary development may vary in different clades (Devlin and Nagahama 1999).

Moreover, the gonadal development can be classified as asynchronous and synchronous. The asynchronous fish have multiple daily spawning and seasonable spawning. In an asynchronous ovary organization, three stages are present during the vitellogenesis. In fact, the oocyte immature egg development is gonotropin-independent and body size-dependent—oocytes of all stages of development are present without dominant population and the ovary appears to be a random mixture of oocytes at every conceivable stage. The synchronous fish are only seasonal spawners—all oocytes develop and ovulate at the same time (Murua and Saborido-Rey 2003).

In mammalian ovaries, the number of oocyte that can be ovulated is limited, because the mitotic division of germ cells is commonly completed before birth and does not occur thereafter. In contrast, in teleosts, there can be an infinite number

of mitotic germ cells, which are histologically observed as oogonia. Thus, the proliferation of oogonia can continuously supply mature eggs throughout life (Nakamura et al. 2011) (Pepling and Spradling 2001) (**Figure 10**). Mitosis can take place in a mature ovary (Tokarz and Jones 1978) and also after birth (Wallace and Selman 1990), whereas in mammals, the embryonic and foetal period are limited (**Figure 10**) (De Felici et al. 2005; Nakamura et al. 2011). Oogenesis, which begins with the oogonia and is followed by development into oocytes, comprises four stages. It begins with the first division (I), undergoes pre-vitellogenesis (II) and vitellogenesis (III), and ends with maturation (IV) (Pavlov, Emel'yanova, and Novikov 2009). During the pre-vitellogenic stage, mainly a growth of the cells takes place, which is largely associated with several morphological changes and their cells are clearly visible in the other stages (Pavlov, Emel'yanova, and Novikov 2009).

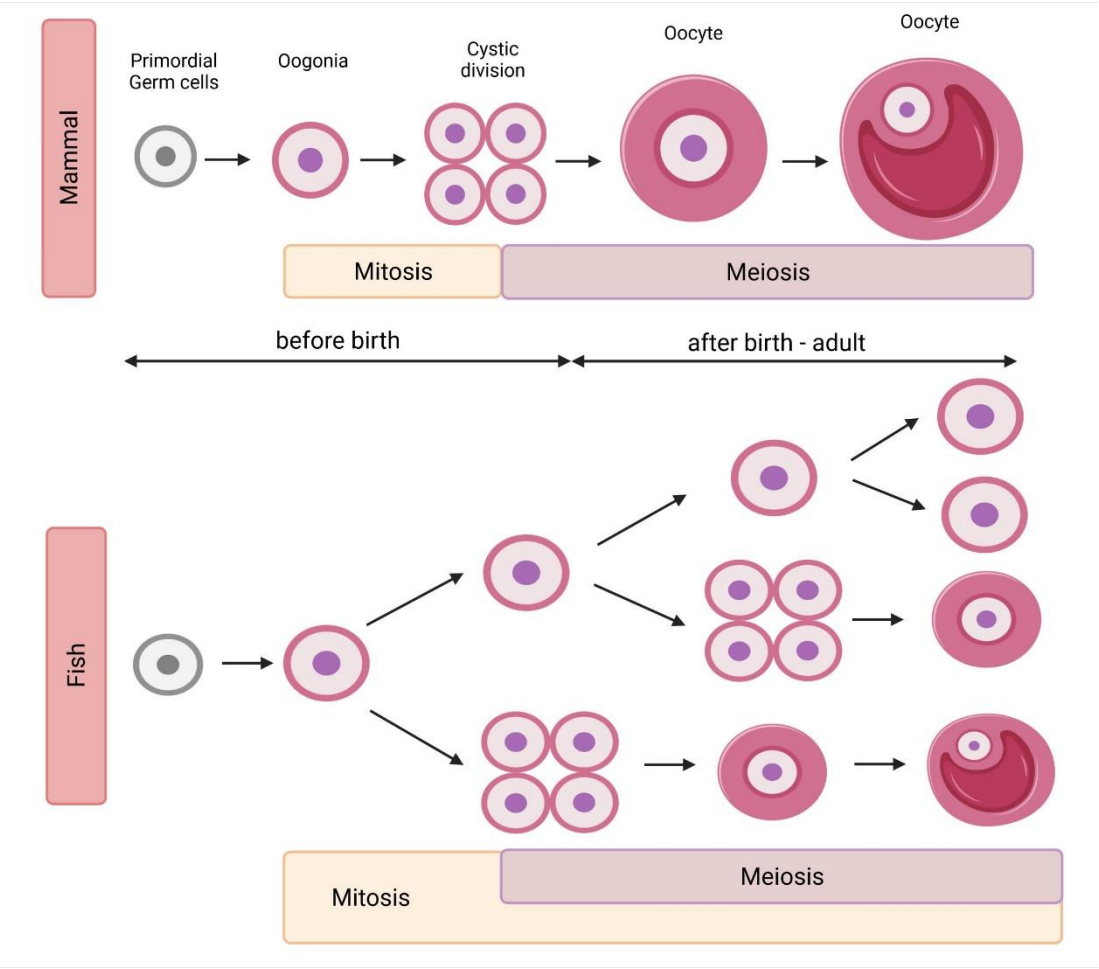

Figure 10: A schematic representation of oogenesis in vertebrate and asynchronon gametogenesis of fish.

Oogenesis in vertebrates: on the first line mammal's oogenesis: mitotic division of oogonia starts before birth and finishes after the first division. Meiotic germ cells are only present in ovary after birth or in adult ovary. Mitotic oogonia are not observed after birth as well. (Pepling and Spradling 2001; Pepling and Spradling 1998). In contrast, the mitosis of fish oogonia was histologically observed also in adult ovary (Wallace and Selman 1990). The schematic presentation of oogenesis in vertebrate is inspired from (Nakamura et al. 2011). Figure created with BioRender.com.

Since mRNA of the polySTs have been detected in fish ovaries and fish eggs contain polySia (Asahina et al. 2006; Sato et al. 1993), it was of particular interest for us to find out during which events of oogenesis polysialylation occurs.

2. Aim and objectives

Sias are the most frequent terminal residue of glycans in vertebrates and they are involved in many essential cellular events (Angata and Varki 2002). Their polymers are very important for the neuronal development and proliferation of neuronal cells. Because of that, the highest level of polySia can be detected on the cell surface of neuronal cells in vertebrates. Interestingly, in mammals, only polymers of α2,8-linked-Neu5Ac residues can be found; in fish eggs, a high variety of different polySia polymers were observed (Inoue and Iwasaki 1978; S. Inoue et al. 1987). Due to the WGD, the two polySTs (ST8Sia II and ST8Sia IV) could have a particular sequence composition and distribution in Actinopterygii, which may explain such a polySia diversity in fish.

Thus, the first objective of this study was the re-evaluation of the polySTs evolutionary relationships and distribution in teleosts to understand this observed polySia diversity.

Since little is known about the role of polySia during oogenesis in fish, the second objective of this study was the investigation of the polysialylation status in the ovaries of two teleost fish with economic relevance on the Baltic Sea area to understand how the polySTs co-ordinately contribute to polySia synthesis during oogenesis.

As polySia is involved in numerous neurological events in mammals (Rutishauser 2008), such as spatial learning (Eckhardt et al. 2000), and memory storage on fear condition, the third objective of this study was to investigate, if the expression of the polySTs is influenced in different fish brain regions after handling stress.

3. Discussion

Glycans of proteins and lipids can be elongated with Sias, which modulate numerous essential processes (Schauer 2004). Besides monosialyl-residues, long polymers can be detected in vertebrates. Remarkably, in fish a high structural diversity of polySia was found (Sato and Kitajima 2013), whereas in mammals, only homopolymers of Neu5Ac were described. Thus, it seems likely that the polysialylation machinery in fish shows different characteristics in comparison to mammals. To this end, we have studied and refined the evolutionary relationships of the Teleost ST8Sia (Harduin-Lepers et al. 2008). We have undertaken bioinformatic strategies including phylogenetic tree construction and synteny/paralogy analyses, which have been proven to be useful not only for the comprehensive identification of vertebrate STs genes, but also for the reconstruction of their evolutionary history. This work led to a first publication (Venuto, Decloquement, et al. 2020).

3.1 Molecular characterization of ST8Sia gene family in fish

All animal sialyltransferases belong to the CAZy glycosyltransferase family GT29 (Lombard et al. 2014; Coutinho et al. 2003). Consequently, this reflects their common origin and organization. To investigate the *st8sia* gene repertoire and its distribution in vertebrates, we used the public databases NCBI, ENSEMBL and Phylofish to collect and discuss the results (Altschul et al. 1997; Pasquier et al. 2016). Due to of the high degree of similarity between the two subfamilies of mammalian *st8sia* sequences, we used only *Homo sapiens* sequences for the initial phylogenetic analysis. We used multiple sequence slignments (MSA) to identify and select the complete open reading frame, while also using the well-known fish sequences, *Lepisosteus oculatus*, *Danio rerio*, and *Oncorhynchus mykiss*. Basic local

alignment search tool (BLAST) (Altschul et al. 1997) strategies were carried out to collect the orthologuous polySTs sequences.

As mentioned earlier, all protein sequences from the GT29 family have the four consensus motifs (L, S, III, VS) (Datta and Paulson 1995; Geremia, Harduin-Lepers, and Delannoy 1997), which are functional signatures of sialyltransferases and were useful for sequence identification in public databases (Datta and Paulson 1997; Drickamer 1993). We identified and analysed a pool of 700 related-sequences from all branches of the animal phylogenetic tree to understand and provide the explication of the teleost repertoire (**Study I, Supplemental Data 1)** (Venuto, Decloquement, et al. 2020). Interestingly, we found multiple copies of *st8sia*-related sequences from different teleosts that had disappeared in tetrapods (**Study I, Supplemental Table S1**) (Venuto, Decloquement, et al. 2020). *In silico* analyses revealed, that the emerging of these families was either prior or after WGD-RND1 and RND2 (**Study I, Figure 2**) (Venuto, Decloquement, et al. 2020). To explain this expansion of *st8sia* genes, we investigated the Neopterygii branches in detail, which are a subclass of Actinopterygii. They were able to evolve faster than their ancestors. Therefore, they became the dominant group of fishes. Neopterygii included also the Teleostei, which compromise the vast majority of fish (López-Arbarello 2012) (**Figure 6**). We created phylogenetic trees and tried to trace back the origin of the two major phylogenetic groups, the mono-α2,8 sialyltransferases and the oligo and poly–α2,8 sialyltransferases (**Study I, Figure 2**) (Venuto, Decloquement, et al. 2020). Intriguingly, some *st8sia* genes seem to be lost during the evolution of some orders (**Figure 11) and (Study I, Table 1)** (Venuto, Decloquement, et al. 2020). For example, *st8sia2* is not present in Siluriformes, Esociformes, and in one family of of Gymnotiformes—*Electrophorus electricus* of Gymonotidae because it diverged from the family Sternopygoidei (Brochu Diss 2011). *St8sia4* is also lost in the Neoteleosteii genome as already described by

Harduin-Lepers and colleagues (Harduin-Lepers et al. 2008). In contrast, two copies of *st8sia4* exist in Cypriniforme (in the Cyprinidae family, *Ciprinus carpio* and *Sinocyclocheilus anshuiensis*) and two copies of *st8sia2* exist in Salmoniformes.

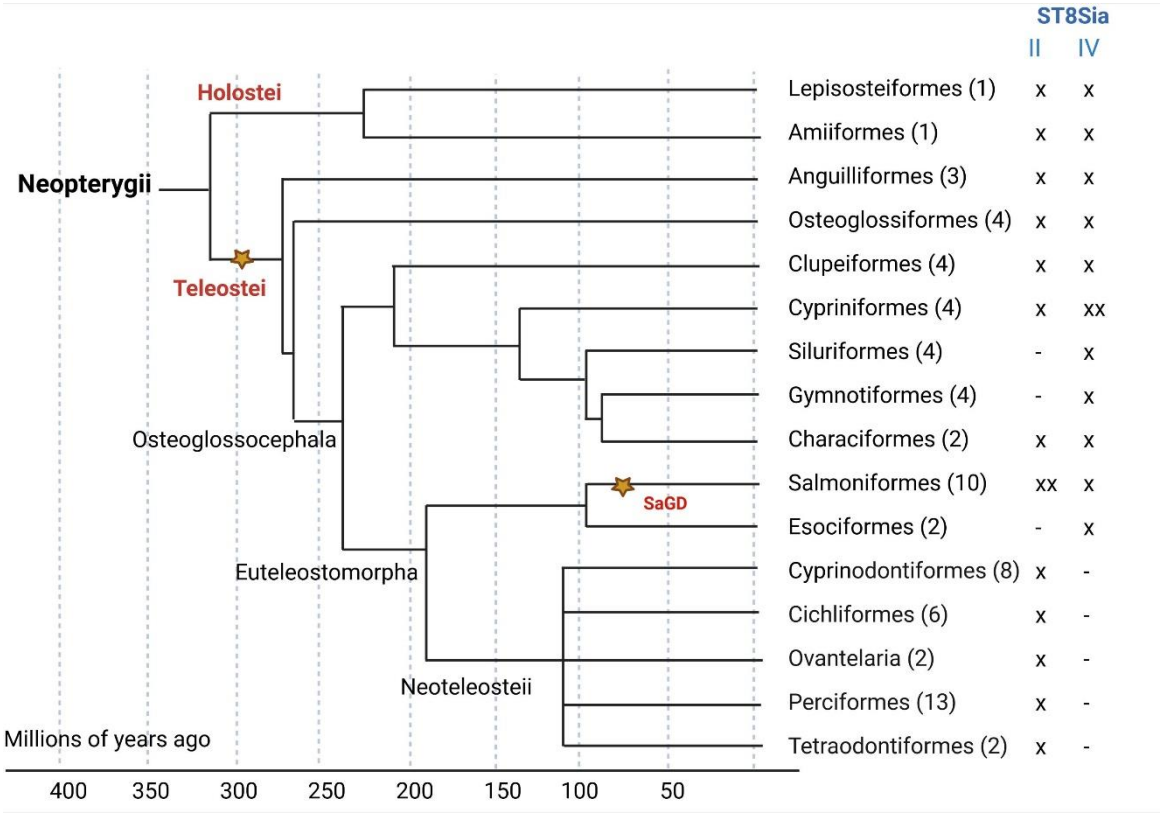

Figure 11 Phylogenic tree of the Neopterygii species.

Cladogram showing phylogenetic relationships among ray finned fish (Actinopterygii) in particular on the subclass Neopterygii. For each phylogenetic group, the number of species is indicated between brackets. **(Study I, supplemental Table 1**) (Venuto, Decloquement, et al. 2020). The Teleost fish specific (TGD) and Salmonidae specific duplication (SaGD) are indicated with the yellow stars. X, presence of ST8Sia; XX, duplicated ST8Sia; - absence of ST8Sia. Figure inspired from (Pasquier et al. 2016; Venuto, Decloquement, et al. 2020). Figure created with BioRender.com.

Furthermore, we identified the two *st8sia2*-related sequences *st8sia2-r1* and *st8sia2-r2* in all Salmonidae (*Oncorhynchus mykiss*, *Coregonus maraena*, *Salvelinus alpinus*, and *Thymallus thymallus*) (**Study I, Supplemental Table S1; Study I, Supplemental Figure 1**) (Venuto, Decloquement, et al. 2020). Interestingly, we observed that these genes are expressed in a tissue-dependent manner in *C.*

maraena (**Study I, Figure 9B)** (Venuto, Decloquement, et al. 2020), which let us to suggest that all variants of the polySTs are functional.

The phylogenetic tree built in that way, shows with its topology, that the duplication in *st8sia* genes is not associated to the teleost-specific duplication, but more with the recent SaDG (Macqueen and Johnston 2014) and the cyprinid duplication (Macqueen and Johnston 2014; Xu et al. 2014; Nacher et al. 2002). Moreover, our phylogenetic studies confirmed, that the two *st8sia2* genes in Salmoniformes are a result of the specific SaDG-RNS4, which occurred about 95 mya (**Figure 12)** (**Study I, Supplemental Figure 1)** (Venuto, Decloquement, et al. 2020).

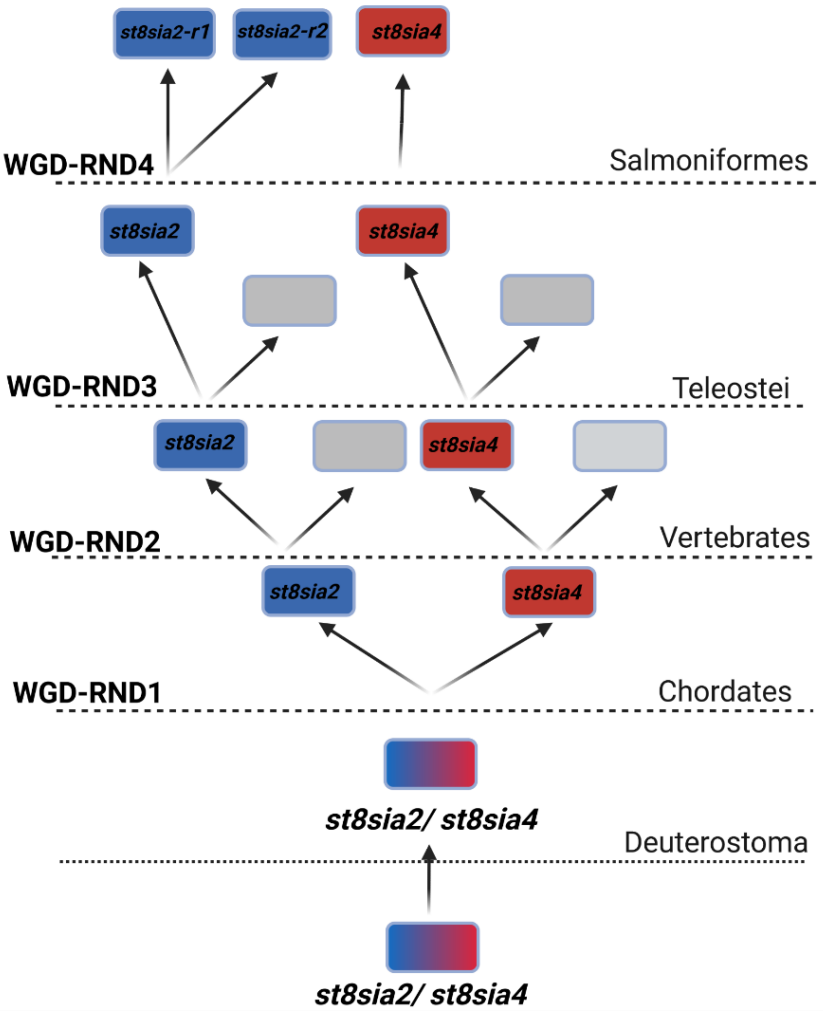

Figure 12: Schematic drawing of the evolution of the polySTs family.

Model of the evolution based on the evidence from protein sequences phylogeny. Grey box represent gene loss following block duplications. The broken lines correspond to main genes duplications events (WGD-RND1 and WGD-RND2). The dotted lines correspond to the intermediate steps. Figure modified from (Venuto, Martorell-Ribera, et al. 2020). Figure created with BioRender.com. CC-BY.

In order to gather and understand the evolutionary history of the *st8sia* families, the determination of orthologies and parologies was another important objective. A well-conserved synteny (order and position of genes on a chromosome) was established on genes loci of the *st8sia2* and *st8sia4* in tetrapods like human, mouse, chicken, and xenopus as well as on an ancient fish, spotted gar (**Study I, Figure 3a**) (Venuto, Decloquement, et al. 2020). Some paralogues of *st8sia* genes were found outside these conserved syntenies on other chromosomes in the same fish. This intense rearrangement could be a consequence of TGD duplication. In Esociformes and Siluriformes, the *st8sia2* gene was lost and only the neighbouring genes could be retrieved in zebrafish and medaka fish genome. In addition, the zebrafish and electric fish *st8sia4*-neighbouring genes were used to recover the genes in Neoteleostei, proving the loss of *st8sia4* in these genomes (**Study I, Figure 3a**) (Venuto, Decloquement, et al. 2020).

The neighbouring genes surrounding *st8sia* genes loci were also found in the Salmonidae family. The synteny around the two *st8sia2-r* gene loci corresponded to the spotting gar orthologous neighbouring genes, indicating the occurrence of a genome duplication in Salmonidae. Similarly, the two *st8sia4-r* gene loci were localized on two paralogous Cypriniformes chromosomes (**Study I, Figure 3a)** (Venuto, Decloquement, et al. 2020). The present results also support the hypothesis of a more recent species-specific genomic event in Cypriniformes (Xu et al. 2014). These synteny studies let us focus on the retained genes after tetraploidization, which are directly involved in complex polySia (heteropolymers) formation.

At the protein sequence level, we hypothesized that the different amino acid substitutions observed in the polyST peptide could influence the interaction with the nascent polySia chain. In other words, structural changes of the protein backbone during the polySTs evolution could be an explanation for the high variations observed in terms of Sia residues in fish compared to mammals (Sato et al. 1993). The fish polyST sequences showed 60% identity compare to mammals sequences (Datta and Paulson 1997). Based on studies of Troy and colleagues (Nakata, Zhang, and Troy 2006), the analysed PSTD motif contain 11 basic residue, Ile (I31), Lys (K32), and Arg (R33) which are the amino acids required for the polysialylation. However, changing Lys (K2), His (H4), or Asn (N6) for instance, may have an impact, since they are involved in the polysialylation (Nakata, Zhang, and Troy 2006). Remarkably, changes in this polybasic domain that occur in phylogenetic fish and enzyme families can affect the attachment of the Sia residue to the new chain during the polysialylation reaction (Sevigny et al. 1998) (**Figure 13**) and (**Study I, Figure 7** and **Figure 8**) (Venuto, Decloquement, et al. 2020).

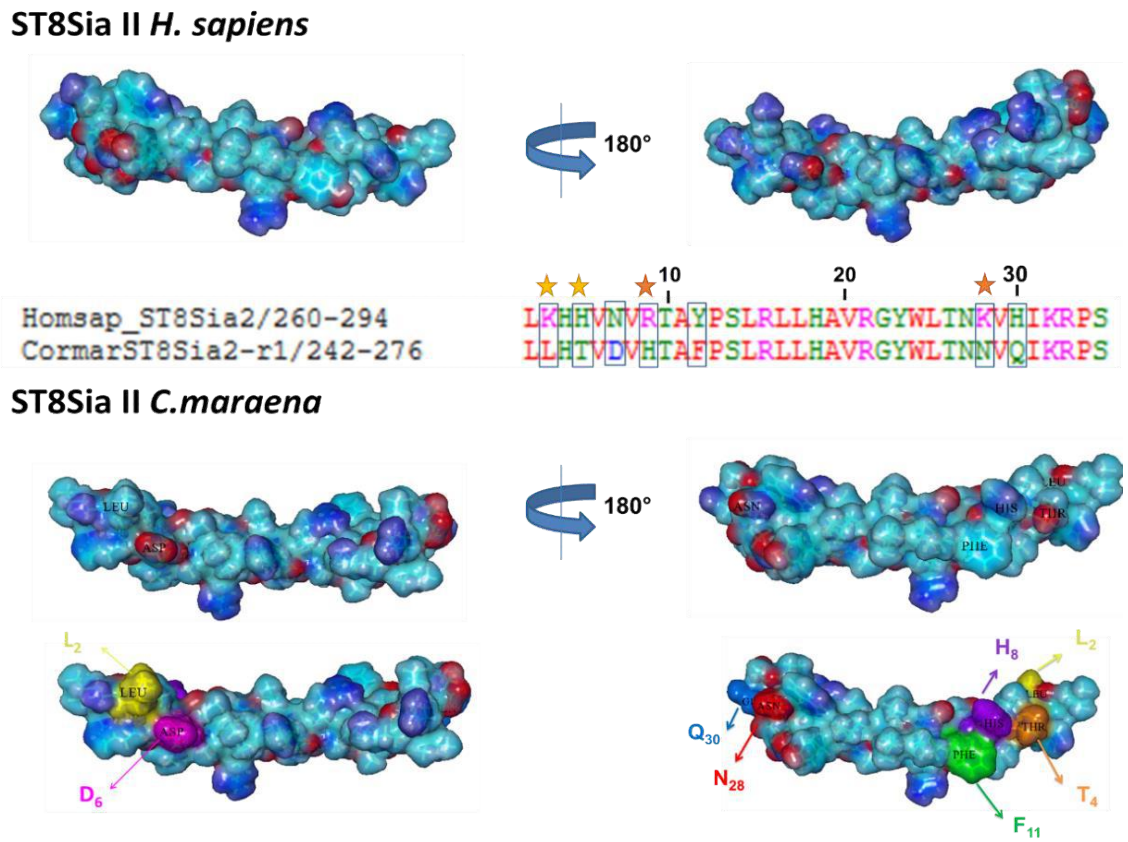

Figure 13: 3D structures of PSTD motif of ST8Sia II

The 3D model of PSTD of ST8Sia II of *Homo sapiens* (*H. sapiens*) (on the top) and the model of PSTD of ST8Sia II of *C. maraena*. Figure created with YASARA. Figure modified from (**Study I, Figure 8**) (Venuto, Decloquement, et al. 2020). CC- BY.

3.2 Polysialylation in fish ovaries

Since polyST mRNA was detected in ovaries (Asahina et al. 2006) and eggs (Sato et al. 1993) of Salmonidae fish, the polysialylation status was analysed in ovaries of *C. maraena*. At first, the ovaries were tested for polySia by Western blotting (WB) using the specific monoclonal antibody (mAb) 735, which recognizes α2,8-linked Neu5Ac polymers. The obtained staining showed the typical smear for polysialylated proteins (Frosch et al. 1985; Nagae et al. 2013) (**Study II, Figure 1a**) (Venuto, Martorell-Ribera, et al. 2020). The immunostaining was verified by a degradation of polySia with an endoneuraminidase (endoN) (Stummeyer et al. 2005), which resulted in a loss of the signal demonstrating that polySia is

synthesized in the ovaries of *C. maraena*. However, the WB cannot be used to determine the composition of the detected polySia chains, since the applied polySia antibody can bind α2,8-linked Neu5Gc or heteropolymers of Neu5Ac and Neu5Gc in addition to homopolymers of Neu5Ac (Naito-Matsui et al. 2017; Davies et al. 2012).

To determine the involved polySTs, qPCR was applied. Since in Salmonidae fish, three distinct polySTs genes are expressed and a seasonal oogenesis takes place (Asahina et al. 2006), we collected samples from summer and autumn and the level of the three polySTs mRNA were analysed. The experiment demonstrated that *st8sia2-r2* exhibit the highest expression level with values approximately 60 times higher than *st8sia2-r1* and *st8sia4.* However, we observed no significant or only minor changes between summer and autumn (**Figure 14**) and (**Study II, Figure 2b/c)** (Venuto, Martorell-Ribera, et al. 2020).

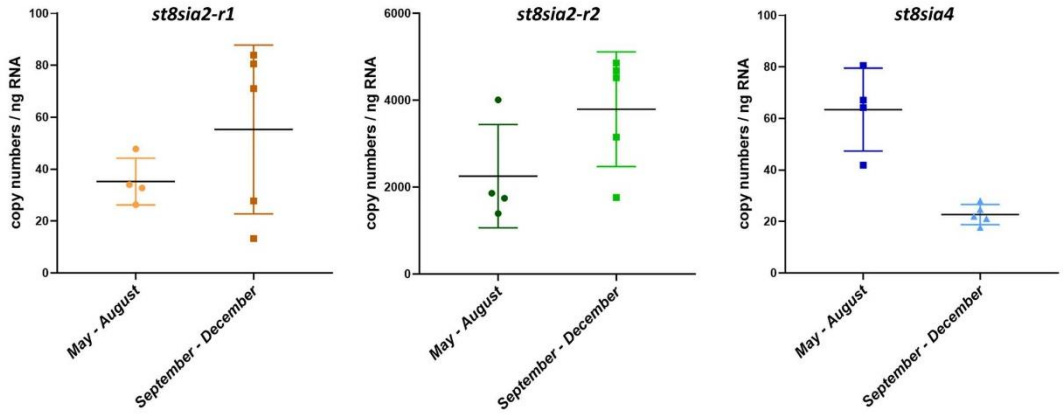

Figure 14: Expression profiling of poly-α2,8sialyltransferases encoding genes in C. maraena ovaries.

Expression profiling representation during summer (n= 4 animals; May - August) and spring (n= 5 animals; September – December). The transcript level of *st8sia2- r1* (orange), *st8sia2- r2* (green), and *st8sia4* (blue) were determined in *C. maraena* ovaries. Data represents the average copy number of the three genes. The error bars represent the

standard deviation. Non-significant, ns; *p <0,05. Figure modified from (Venuto, Martorell-Ribera, et al. 2020) CC-BY.

To localize the origin of polySia, the ovaries were analyzed by immunohistochemistry using mAb 735 against polySia. The examination of the histological section from *C. maraena* revealed, that the main polySia positive cluster of cells exists during the pre-vitellogenic stage. Comparable with the WB staining **(Study II, Figure 1a/1b)** (Venuto, Martorell-Ribera, et al. 2020), the signal was verified by a degradation of polySia with endoN **(Figure 15)** and **(Study II, Figure 4a/4b; Study II, Supplemental Figure 2a)**

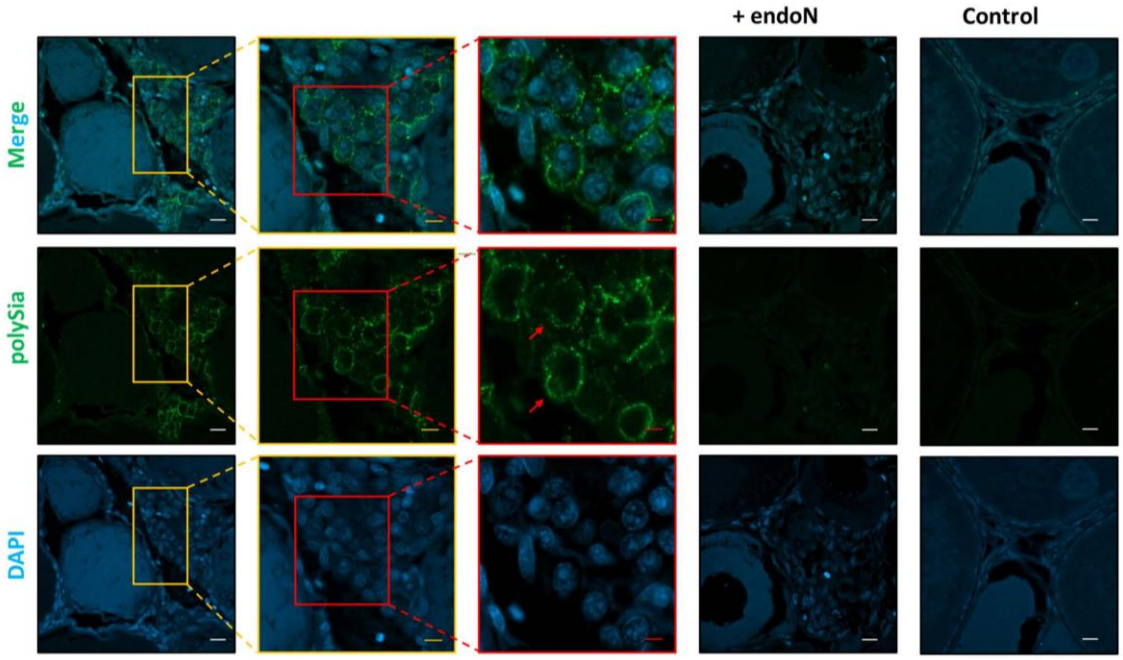

Figure 15: Histological analysis of polysialylation status of C. maraena ovary

For the immunohistochemical analysis, we used mAb 735 combined with Alexa Fluor 488-conjugated secondary antibody (green) to detect polySia. Negative control was performed omitting the first antibody. For supplementary negative control, polySia was degraded by endoN (+endoN). Red arrows showing the selected polySia-positive cells. Nuclei were stained using DAPI (blue). Scare bars: 20µm (white), 10µm (orange), and 5µm (red) (Venuto, Martorell-Ribera, et al. 2020) CC-BY.

Interestingly, Nakamura and colleagues described such cell clusters in *Oryzia latipes* ovaries, which seems to be nest of oogonia (Nakamura et al. 2011). Based on

this study, it was interesting to localize oogonia and polySia in parallel. The localization of oogonia nest on the *C. maraena* ovary tissues were performed using an antibody against Aspartic acid (D)-Glutamic acid (E)-Alanine(A)- Aspartic acid (D) helicase gene 4 (DEAD-box helicase gene 4, DDX4) (Clarkson et al. 2019). The result demonstrates that DDX4-population are frequently polySia positive (**Study II, Figure 5a and 5b**) (Venuto, Martorell-Ribera, et al. 2020).

We highlighted that in Neoteleostei (*S. lucioperca*), ST8Sia IV is lost and the only polySTs is ST8Sia II (**Study II, Figure 6a/6b** and **Supplemental Figure 1**) (Venuto, Martorell-Ribera, et al. 2020). In according with this, we carried out an investigation of the potential functional consequences of lost genes on the remaining gene expression and function. Quantitative analysis of the ST8Sia II from *S. lucioperca* have shown that the expression level of the gene is higher compared to the more expressed *st8sia* in Salmonidae ovary (**Study II, Figure 2** and **Figure 6c**) (Venuto, Martorell-Ribera, et al. 2020). The obtained results suggest that the Percidae *st8sia2* could have the same function of three *st8sia* as on Maraena whitefish because of the co-orthologues function (**Study I, Figure 6b**) (Venuto, Martorell-Ribera, et al. 2020). Moreover, we explored the polysialylation status of *S. lucioperca* ovaries with the same procedure as for *C. maraena.* Ovarian tissue sections from *S. lucioperca* were stained in parallel using antibodies against polySia and DDX4. Similar to *C. maraena*, clustered cell populations were polySia positive (**Study II, Figure 7** and **Figure 8***)* (Venuto, Martorell-Ribera, et al. 2020). In both fish, the polySia positive cells are located on the peripheral connective tissues that contain ovarian follicles. In this part, most of the cells are oogonia and early-stage oocytes. The oogonia are clearly distinguishable by their round shape, size, and typical spherical nucleus. The results of co-localization of oogonia for *C. maraena* and *S. lucioperca* suggest that oogonia might be one source of polySia in the analysed ovaries (**Study II, Figure 5** and **Figure 8)** (Venuto, Martorell-Ribera, et al.

2020). However, more *in vivo* studies have to be performed to understand their physiological role in oogenesis in vertebrates.

3.3 Physiological/neurological functions of polySia

In vertebrates, polySia is most strongly expressed during the development of the nervous system. In the first weeks of postnatal development, polySia vanishes almost completely from the brain. However, there are some regions in the adult vertebrate brain where polySia is still present, particularly where neuronal plasticity or regeneration occurs. For instance, polySia has been detected in mammals, in a subpopulation of mature interneurons in the hippocampus (Seki and Arai 1993) (Nacher et al. 2002) but also in the amygdala, prefrontal cortex, and hypothalamus (Bonfanti et al. 1992). The brain of teleost fish is commonly divided into following parts: forebrain (FB) (amygdala, hippocampus), midbrain (MB) (thalamus and hypothalamus), and hindbrain (HB) (cerebellum) (Mueller 2012; Winberg and Nilsson 1993) and (**Study III, Figure 1**)(Martorell-Ribera et al. 2020).

As previusly mentioned, polySia is important for the hippocampus-related synaptic plasticity, such as spatial learning (Eckhardt et al. 2000) and memory storage on fear condition (water maze) (Markram, Gerardy-Schahn, and Sandi 2007). In addition, it has been shown that acute stress induced a decrease in polySia expression (Chikara et al. 2019). Based on these studies, the effect of a short acute stress handling (**Study III, Figure 2**) (Martorell-Ribera et al. 2020) on the expression of polySTs was investigated in different brain sections of *C. maraena*. The polySTs were analysed in three-selected sections of the brain (**Figure 16 unpublished data**). We detected moderate expression for *st8sia2-r1* in the FB. Expression in the MB is weak and marginally detectable in the HB. *St8sia2-r2* is expressed significantly weaker than the *St8sia2-r1* form in the forebrain and is hardly detectable in the midbrain and hindbrain. In the adult human brain, in contrast, *st8sia2* mRNA is

mainly expressed in the pons and medulla, a region corresponding to the hindbrain of fish (Uhlén et al. 2015).

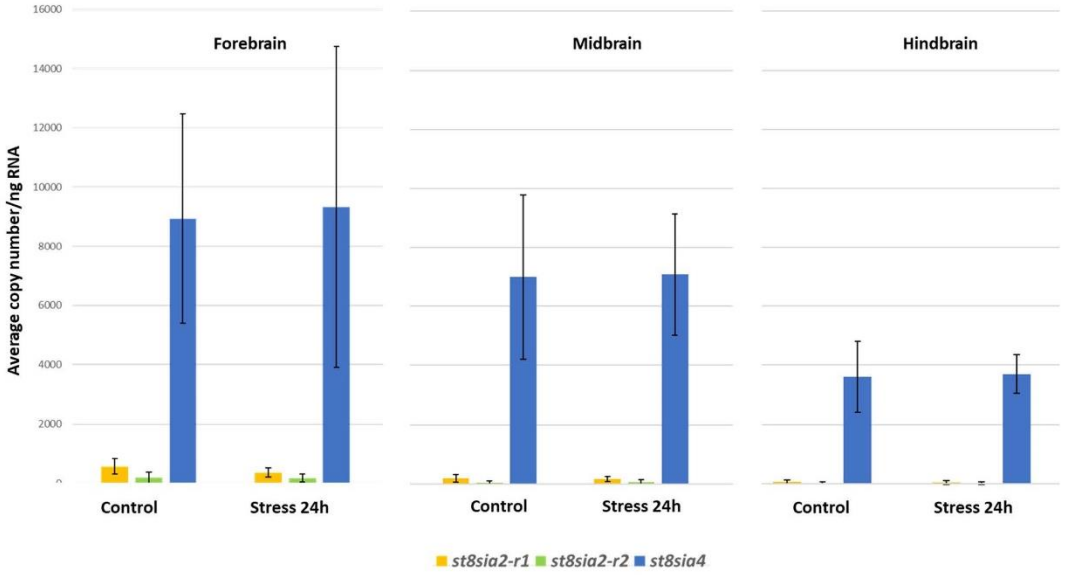

Figure 16: Expression profiling of STs – encoding genes in C. maraena brain.

Transcript level of the polySTs were determinate in the three brain tissues forebrain, midbrain and hindbrain of *C. maraena* (n=5 control and n=5 stressed). Expression of the control and stressed fish after 24h handling are indicated on the abscissa. Expression analyses were performed using the brain tissues from Study III (Martorell-Ribera et al. 2020). The oligonucleotide primers used are the same used on Study I (Venuto, Decloquement, et al. 2020) and II (Venuto, Martorell-Ribera, et al. 2020) for the expression profiling of polySTs. The corresponding colored bars represent the averaged (media) of the copy numbers/ng RNA, and errors bars represent the standard deviation (min and max). Orange bars (*st8sia2-r1*), green bars (*st8sia2-r2*), and blue bars (*st8sia4*).

Although ST8Sia IV is the predominant polyST in adult human tissues, its expression level and pattern in the adult human brain is comparable to ST8Sia II (Uhlén et al. 2015). In contrast, we found that in *C. maraena st8sia4* is by far the most dominant polyST in all three parts of the brain we examined with lower expression in the HB. In our experiment, the expression profiling of polySTs was similar before and after stress treatment. It should be mentioned that we observed strong animal-individual variations in the expression level of STs. However, our results are comparable to a study of mouse brain after acute stress, where the authors

found that the expression of polySTs did not change despite polySia expression being reduced. This is due to the stress-induced activation of α2-3,6-sialidases (Chikara et al. 2019). To understand the kinetics of the polySTs expression in the brain during handling stress, more experiments should be performed. Furthermore, examination of polySia expressing cells on the brain tissues at different time after stress could provide deeper insight into these phenomena.

Besides expression levels of polySTs, we analysed several other classical stress markers. Thereby, we could detect alterations especially in the monoamine-influenced (noradrenalin [NA], dopamine [DA], and serotonin [5-HT]) systems (**Study III, Figure 3, A–B)** (Martorell-Ribera et al. 2020). Monoamines play an important role in coping with stress (Winberg and Nilsson 1993), and interestingly, polySia is involved in dopamine (DA) signalling (Angus et al. 1982). Moreover, the binding feature of polySia is involved in the brain function because of the interactions of the polySTs (Calandreau et al. 2010), likely via the catechol backbone (Isomura, Kitajima, and Sato 2011). DA and other monoamine neurotransmitters were used as sensitive markers of indicators of stress response in fish (Chabbi and Ganesh 2015). The results obtained here have shown that the effect of the stress has repercussions on different regions of the brain after acute handling and let us hypothezise that the acute handling of stress slightly influences likely the dopaminergic genes, which may be indicator of habituation (Martorell-Ribera et al. 2020). However, to investigate the role of polySia during such stress events, more detailed *in vivo* studies have to be performed.

4. Conclusion

The outlined studies demonstrate that during teleost evolution several changes of the polysialylation machinery took place. For instance, in Salmoniformes, the *st8Sia2* gene was duplicated leading to two *st8sia2* genes, whereas in Neopterygii a loss of *st8Sia2* occurred. In addition, several differences in comparison to mammals were observed when the amino acid sequences of the catalytic domain were compared, which may explain the high polySia diversity in fish. The expression of polysialyltransferases in several tissues as well as the presence of polySia on oogonia, suggest that in fish, sialic acid polymers are involved in additional physiological mechanisms other than the known one in mammals.

5. Publications

5.1 Study I: Vertebrate Alpha2,8-Sialyltransferases (ST8Sia): A Teleost Perspective

Venuto, M.T.; Decloquement, M.; Martorell Ribera, J.; Noel, M.; Rebl, A.; Cogez, V.; Petit, D.; Galuska, S.P.; Harduin-Lepers, A. *Int. J. Mol. Sci.* **2020**, *21*, 513. (https://doi.org/10.3390/ijms21020513)

Brief summary:

In vertebrates, the ST genes represent a multigene super-family of homologous sequences (CAZy GT family 29), either orthologues or paralogues. The BLAST screening in the NCBI database suggests an expansion of the ST8Sia family in fish and during the most recent years, bioinformatics strategies have been developed for the comprehensive identification of vertebrate ST genes. The aim of the study was, to identify and analyse the α2,8-sialyltransferases sequences among the ray-finned fish to create the teleost St8sia repertoire. The undertaken bioinformatics strategies have provided useful analyses, not only for the comprehensive identification of vertebrate *st8sia* genes but also for the reconstruction of their evolutionary history with phylogenetic tree construction and synteny/paralogy analyses. Their evolutionary relationships assessed around the teleost *st8sia* loci provide relevant and solid information that enabled us to reconstruct the evolutionary history of *st8sia* genes in Teleostei genomes. Our data also indicates that the fish α2,8-sialyltransferase family appears to be much more diverse than the mammalian ST8Sia family, which is comprised of 6 subfamilies forming di-, oligo-, or polymers of α2,8-linked Sias. Additionally, the fish α2,8-sialyltransferase family shows a wide scattered distribution among fish species. MSA led us to identify conserved α2,8-polySTs sequence motifs and to detect changes in the conserved PSTD domain of the various fish that could account for variable polyST activities.

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Article

Vertebrate Alpha2,8-Sialyltransferases (ST8Sia): A Teleost Perspective

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Abstract: We identified and analyzed α2,8-sialyltransferases sequences among 71 ray-finned fish species to provide the first comprehensive view of the Teleost ST8Sia repertoire. This repertoire expanded over the course of Vertebrate evolution and was primarily shaped by the whole genome events R1 and R2, but not by the Teleost-specific R3. We showed that duplicated *st8sia* genes like *st8sia7*, *st8sia8*, and *st8sia9* have disappeared from Tetrapods, whereas their orthologues were maintained in Teleosts. Furthermore, several fish species specific genome duplications account for the presence of multiple poly-α2,8-sialyltransferases in the Salmonidae (ST8Sia II-r1 and ST8Sia II-r2) and in *Cyprinus carpio* (ST8Sia IV-r1 and ST8Sia IV-r2). Paralogy and synteny analyses provided more relevant and solid information that enabled us to reconstruct the evolutionary history of *st8sia* genes in fish genomes. Our data also indicated that, while the mammalian ST8Sia family is comprised of six subfamilies forming di-, oligo-, or polymers of α2,8-linked sialic acids, the fish ST8Sia family, amounting to a total of 10 genes in fish, appears to be much more diverse and shows a patchy distribution among fish species. A focus on Salmonidae showed that (i) the two copies of *st8sia2* genes have overall contrasted tissue-specific expressions, with noticeable changes when compared with human co-orthologue, and that (ii) *st8sia4* is weakly expressed. Multiple sequence alignments enabled us to detect changes in the conserved polysialyltransferase domain (PSTD) of the fish sequences that could account for variable enzymatic activities. These data provide the bases for further functional studies using recombinant enzymes.

Keywords: molecular phylogeny; α2,8-sialyltransferases; polySia motifs; evolution; ST8Sia; functional genomics

1. Introduction

Glycoproteins and glycolipids can be modified with numerous different glycans during their transit to the cell surface. Here, these glycoconjugates form a dense meshwork, the glycocalyx, influencing several essential processes, such as adhesion and migration mechanisms in addition to

cell signaling. Intriguingly, all living cells are surrounded by such a sugar-coat, which demonstrates the importance of glycans for all living organisms [1]. However, glycoconjugates are not only found on the cellular membranes, but also on released extracellular vesicles and soluble glycoconjugates; likewise, various physiological and pathological can be targeted by their released forms. Several different monosaccharides are utilized for the formation of glycans. Nevertheless, a very special position among the building blocks of glycans takes the family of sialic acids [2,3]. These α-keto acids consist of a nine-carbon backbone with a carboxylic acid group at C1 and a ketone group at C2 [4]. Remarkably, more than 50 derivatives are known in nature. Besides N-acetylneuraminic acid (Neu5Ac), N-glycolylneuraminic acid (Neu5Gc) is the most common sialic acid and the hydroxyl groups of both can be additionally substituted, for example, by acetylation. The same applies for a further common sialic acid, which is mainly used in lower vertebrates, deaminated neuraminic acid (KDN, 2-keto-3-deoxy-D-glycero-D-galacto-nononic acid) [5]. All three of these sialic acids are frequently added by α2,3- and α2,6-sialyltransferases (ST3Gal, ST6Gal and ST6GalNAc) to nascent glycans. However, in contrast to other commonly utilized monosaccharides of glycans, an attached sialic acid residue can only be used to add another sialic acid residue, which explains their outermost position on sialylated glycans. The elongation at position C8 of α2,3- or α2,6-linked sialic acid residues is catalyzed by sialyltransferases belonging to the group of α2,8-sialyltransferases (ST8Sia) and long polymers of sialic acids can be enzymatically synthesized in this way [6–8].

All those animal sialyltransferases (α2,3-, α2,6- α2,8-sialyltransferases) belong to the CAZy glycosyltransferase family GT29, which indicates their common modular organization (GT-A-like fold) and their common ancestral origin [8,9]. These protein sequences are characterized by the presence of four consensus motifs called sialylmotifs (L (Large), S (Small), III, and VS (Very Small)) involved in 3D structure maintenance, substrate binding, and catalysis [10,11]. The sialylmotifs are very useful for in silico identification of sialyltransferases-related sequences [12]. On the basis of their sugar acceptor specificity and glycosidic linkage formed, GT29 is subdivided into four families ST3Gal, ST6Gal, ST6GalNAc, and ST8Sia in vertebrates [7,13], each of which is characterized by family motifs likely involved in linkage specificity [14–16]. The biosynthesis of α2,8-sialylated molecules is an ancient pathway achieved by the ST8Sia, a group of enzymes that emerge in the first eukaryotes [8] and expanded very early in animal evolution [14]. Up to now, the ST8Sia enzymes have been studied and characterized in mammalian tissues and primarily in the adult brain. The human and mouse genomes show six ST8Sia subfamilies: ST8Sia I, ST8Sia V, and ST8Sia VI are mono-α2,8-sialyltransferases and constitute a first group of ST8Sia enzymes involved in di-sialylation of glycoconjugates, while ST8Sia III in addition to ST8Sia II and ST8Sia IV form a second group of oligo- and poly-α2,8-sialyltransferases implicated in the polysialylation of glycoproteins [15].

Interestingly, our recent studies pointed to the fact that the *st8sia* gene family appears to be much larger in teleost fish genomes [14,17]. The emergence of several novel vertebrate mono-α2,8-sialyltransferases subfamilies like ST8Sia VII and ST8Sia VIII was described in this first group of ST8Sia and their enzymatic specificities remain to be determined. These mono-α2,8-sialyltransferase genes have arisen as a consequence of whole genome duplications (WGDs, R1 and R2) at the base of vertebrates and were maintained in fish, whereas some others such as *st8sia6*, maintained in Tetrapods, have disappeared in fish [17,18]. In the second ST8Sia group, the enzymes responsible for the biosynthesis of sialic acid polymers, the poly-α2,8-sialyltransferases ST8Sia II and ST8Sia IV and the oligo- α2,8-sialyltransferase ST8Sia III, have been cloned and characterized from mammalian tissues, essentially the brain, where they act on α2,3-sialylated N-glycans of the neural cell adhesion molecule (NCAM), leading to an increased neuronal plasticity in embryos [19–26]. From a structural point of view, the poly-α2,8-sialyltransferases share a high degree of similarity in their sequence and structure [27–29] and are characterized by two additional sequence motifs, termed the polysialyltransferase domain (PSTD), of 32 amino acids located upstream of the sialylmotif S, and the polybasic region (PBR), of 35 amino acids located in the stem region of the enzymes involved in protein-specific polysialylation [30,31]. The oligo-α2,8-sialyltransferases ST8Sia III also show additional broadly conserved motifs with respect to ST8Sia II and ST8Sia IV (motifs III-1 and III-2) [14] with potential implication in the

oligosialylation activity [32]. Their fish orthologues have been identified, cloned, and characterized in zebrafish (*Danio rerio*) in addition to rainbow trout (*Oncorhynchus mykiss*) [18,33,34].

Our previous phylogenetic studies also identified novel α2,8-sialyltransferases-related sequences like the ST8Sia III-related (ST8Sia III-r) found in a few fish orders like Perciformes, Tetraodontiformes, and Beloniformes, whereas the ST8Sia IV disappeared from the Neognathi fish [14]. It has long been appreciated that gene-, segmental-, and genome duplication, as well as gene loss events, have played important role in evolution, providing new genetic materials, which may facilitate new adaptation for the organism [35,36].

In this study, we used a BLAST strategy to identify over 700 ST8Sia-related sequences from ray-finned fish genomes and performed phylogenetic analyses and sequences alignments to reevaluate their evolutionary relationships and fate, focusing on those responsible for polysialic acid (polySia) biosynthesis with implications for the evolution of nervous system, immunological system, and cell–cell interactions. Our findings point to a particular distribution of ST8Sia in fish, revealing novel *st8sia* gene members and further suggesting their functional divergence in vertebrates.

2. Results and Discussion

2.1. In Silico Identification and Phylogenetic Reconstruction of ST8Sia Sequences

To investigate *st8sia* genes' expansion and distribution in vertebrates, we performed public database screenings in the National Center for Biotechnology Information (NCBI), ENSEMBL, and Phylofish databases [37] using a BLAST strategy [38]. The obtained results led to the identification of more than 700 ST8Sia-related sequences (Supplemental Data 1) in chordate genomes, including 71 ray-finned fish genomes (68 Teleosts genomes). Putative ST8Sia sequences with significant similarity to the known human ST8Sia based on the presence of the sialylmotifs L, S, III and VS found in all GT29 sialyltransferases, and of family motifs characteristic for the ST8Sia family were selected, and multiple sequence alignments were performed to select the complete open reading frame. The orthologues of ST8Sia I and ST8Sia V involved in gangliosides biosynthesis are identified in all the investigated genomes, suggesting a high conservation of the gangliosides biosynthetic pathways in vertebrates (Supplemental Table S1). Similarly, the ST8Sia III and the recently described fish ST8Sia VIII [17] could be found in all the Actinopterygii (ray-finned fishes) genomes (Figure 1; Supplemental Table S1). Intriguingly, multiple copies of *st8sia*-related gene sequences were identified in Teleost genomes and their number varied considerably from one fish order or species to another. For example, there are 6 *st8sia*-related genes in the medaka (*Oryzias latipes*), 8 in the clownfish (*Amphiprion oscellaris*) and the common carp (*C. carpio*), and up to 10 in the rainbow trout. Indeed, multiple copies of ST8Sia VIII (>3) were found in Perciformes, Cichliiformes, and Cyprinodontiformes; that is, two copies of the ST8Sia VII in Cypriniformes, two copies of the ST8Sia II in Salmoniformes, and two copies of the ST8Sia IV were found in the Cypriniforme *C. carpio* (Supplemental Table S1). In addition, some other *st8sia* genes could not be found like ST8Sia VI in Teleosts and Chondrostei [17]; ST8Sia IV in Neoteleostei genomes [14]; or ST8Sia II in Esociformes, Siluriformes, or Gymnotiformes (except *Electrophorus electricus*) genomes (Table 1). This resulted in a particular distribution of ST8Sia observed in the Actinopterygii compared with the Sarcopterygii (lobbed-finned fishes and Tetrapods) and Chondrichtyes (sharks) (Figure 1; Supplemental Table S1), which might have facilitated the acquisition of evolutionary innovations during vertebrate evolution [35]. These observations prompted us to re-examine the genetic events, which have shaped α2,8-sialylation in Teleosts.

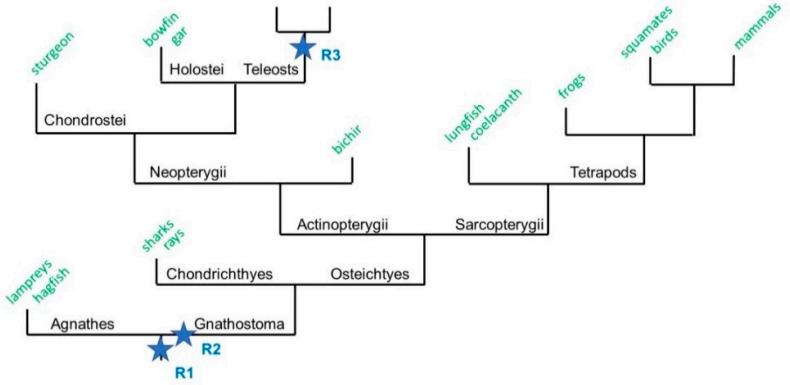

Figure 1. A schematic phylogenetic tree of vertebrate evolution. A simplified phylogenetic tree depicting the evolution of the jawed vertebrates Gnathostomes after the two rounds of whole genome duplication (WGD, R1 and R2). It is hypothesized here that WGD-R2 occurred after the Gnathostomes-Agnathes (jawless vertebrates) split. The Gnathostomes branch is divided into two categories: the cartilaginous fish Chondrychtyes (sharks and rays) and the bony fish Osteichthyes. The Osteichthyes are split into the lobe-finned fish Sarcopterygii that contain Tetrapods, and the ray-finned fish Actinopterygii that contain Neopterygii (Chondrostei, Holostei, and Teleosts).

Table 1. Fish orders that have lost *st8sia* genes.

Missing Sialyltransferase	Fish Order
st8sia2	Siluriformes, Gymnotiformes, Esociformes
st8sia4	Perciformes, Tetraodontiformes, Beloniformes, Cichliiformes, Cyprinodontiformes, Gadiformes, Gobiiformes, Pleuronectiformes, Anabantiformes, Syngnathiforme, Synbranchiformes
st8sia9	Cypriniformes, Siluriformes, Clupeiformes, Gymnotiformes, Characiformes, Lepisosteiformes, Amiiformes
st8sia7	Osteoglossiformes, Cichliiformes, Clupeiformes, Cyprinodontiformes, Gadiformes, Gobiiformes, Pleuronectiformes, Siluriformes

To determine whether the expansion of *st8sia* genes observed in Actinopterygii could be associated to WGD or smaller scale duplication events, we took advantage of the improved genome sequencing of several critical species for basal Vertebrates as Agnathans (Lampreys and Hagfish) and for Actinopterygii as Chondrostei (Sturgeons) and Holostei (Gars and Bowfin) (Figure 1). A simplified dataset was constructed including sequences of Agnathans (*Lethenteron camtschaticum*, *Petromyzon marinus*, *Eptatretus burgii*), Chondrichthyans (*Callorhinchus milii*, *Squalus acanthias*, and *Heterodontus zebra*), basal Actinopterygians (*Acipenser sinensis*, *Amia calva*, and *Lepisosteus oculatus*) and basal Teleosteans such as the Elopomorphs *Anguilla anguilla* and *Mastacembelus armatus*, in addition to two Teleosts, the Beloniforme *O. latipes* (medaka) and the Characiforme *Astyanax mexicanus* (cave fish). The potential orthology of the selected sequences was assessed through the construction of phylogenetic trees (Figure 2). The topology of these trees indicated two major phylogenetic groups of mono-α2,8-sialyltransferases on one hand, and oligo- and poly-α2,8-sialyltransferases on the other, as previously described [7,14].

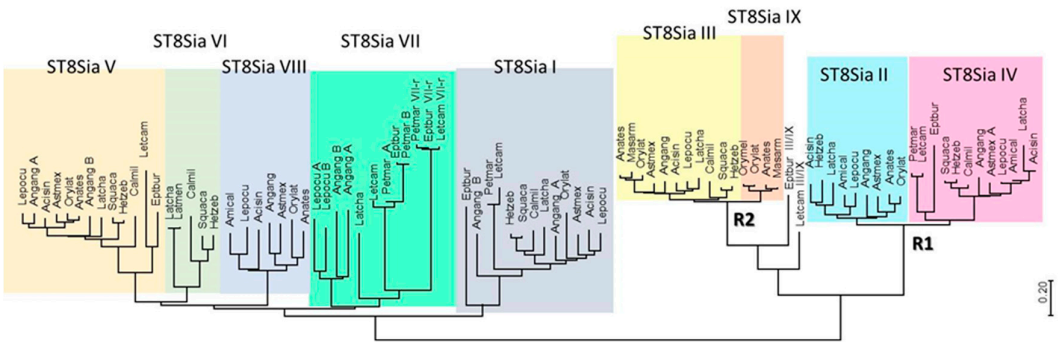

Figure 2. Minimum evolution phylogenetic tree of 89 chordates ST8Sia. The evolutionary history of 89 ST8Sia (see names and sequences in Supplemental Data 1) was inferred using the minimum evolution (ME) method. The optimal tree drawn to scale with the sum of branch length = 16.02931149 is shown. The evolutionary distances were computed using the JTT (Jones-Taylor-Thornton) matrix-based method and the rate variation among sites was modeled with a gamma distribution (shape parameter = 5). The ME tree was searched using the close-neighbor-interchange (CNI) algorithm at a search level of 1. The neighbor-joining algorithm [39] was used to generate the initial tree. The analysis involved 89 amino acid sequences and all positions with less than 95% site coverage were eliminated. A total of 226 positions were in the final dataset (see multiple sequence alignments in Supplemental Data 2). Evolutionary analyses were conducted in MEGA7.0 [40]. The nine Vertebrate subfamilies of ST8Sia (ST8Sia I to ST8Sia IX) are indicated by various colors.

In the mono-α2,8-sialyltransferases group, a series of Agnathan sequences are found at the base of each of ST8Sia I and ST8Sia V. The results corroborate previous findings suggesting the emergence of these two subfamilies around 596 and 563 million years ago (MYA), well before vertebrates emergence and prior WGD R1 and R2 [14]. Consistent with our previous data [17], we identified *st8sia7* genes in the jawless vertebrates *Lethenteron camtschaticum*, *Petromyzon marinum*, and *Eptatretus burgeri* genomes. Thus, these genes might have arisen from the ancestral *st8sia6/7/8* gene after the first WGD R1 event (~552 MYA), although timing of these events with respect to the divergence of agnathans is still a matter of debate [41,42]. Interestingly, Agnathans possess two copies of this later enzyme, named ST8Sia VII and ST8Sia VII-r in Figure 2, likely resulting from species specific large-scale gene duplication events. Similarly, in Teleosts, the eel *A. anguilla* (Elopomorphes, see the work of [43]) also harbors two copies of ST8Sia VII, ST8Sia I, and ST8Sia V enzymes (Figure 2). This observation is in favor of a large-scale genome duplication event different from the Teleost specific third round of WGD R3 (TGD) [44,45], which may have taken place in a common ancestor of freshwater eels sometime after the split of Elopomorpha and Osteoglossomorpha [46]. The ST8Sia VI and ST8Sia VIII subfamilies likely have arisen from the second WGD at the base of Vertebrates; the first one was maintained in Sarcopterygii and disappeared in Actinopterygii, and vice versa for ST8Sia VIII [17]. The many gene copies of *st8sia7* and *st8sia8* identified in Teleosts genomes (Supplemental Table S1) are likely the result of single gene duplication events because they were identified on the same piece of chromosome (data not shown), and were thus noted with -A, -B, or -C extension. However, it is difficult to infer the origin of these segmental duplications as they have occurred in many, but not all terminal branches of clades.

The second branch encompasses both oligo- and poly-α2,8-sialyltransferases. Regarding poly-α2,8-sialyltransferases, the Agnathan sequences were attributable only to ST8Sia IV, indicative of a divergence between ST8Sia II and ST8Sia IV dating back to WGD-R1 (Figure 2) [14] followed by *st8sia2* gene loss in Agnathans. In contrast, the Agnathan sequences of oligo-α2,8-sialyltransferases are at the base of the ST8Sia III and ST8Sia III-r subfamilies, while there are orthologues to the ST8Sia III from sharks to Tetrapod lineages, suggesting a genome duplication event linked to WGD-R2 consistent with previous dating around 474 MYA [14]. Despite the fact that the ST8Sia III-r sequences appear to be restricted to Teleosteans, including Elopomorphes, and are lost in

Chondrichthyans and Tetrapods lineages, they were not issued from the Teleost specific WGD, and thus were renamed ST8Sia IX according to the previously described nomenclature [12].

2.2. Identification and Phylogenetic Analysis of the Fish St8sia Genes (st8sia2, st8sia4, st8sia3, and st8sia9)

Interestingly, in the oligo- and poly-α2,8-sialyltransferases group, the ST8Sia II and ST8Sia IV appeared to be duplicated or lost in several Teleost lineages after divergence of Actinopterygii from Sarcopterygii [47,48], whereas the ST8Sia III was found in all the Actinopterygii. In the basal Elopomorphes and Osteoglossiformes branches, the four *st8sia* genes (*st8sia2*, *st8sia3*, *st8sia4*, and *st8sia9*) could be identified. The results indicate that these genes already existed in the common ancestor of the 68 Teleost fishes examined. All Otocephalan lineages lack the *st8sia9* gene and the Siluriformes lack both the *st8sia9* and *st8sia2* genes. Consequently, the *st8sia9* gene was lost shortly after Otocephala emergence around 176.2 MYA and the *st8sia2* gene was lost more recently (~82.6 MYA) during siluriformes evolution [49]. As previously observed, all Neoteleostei fish lack the *st8sia4* gene [14], which was lost at the basis of Neoteleostei lineage. Finally, the Esociformes lack the *st8sia2* gene only (Table 1). Furthermore, two ST8Sia II-related sequences were identified in all the investigated Salmoniformes (*Oncorhynchus*, *Coregonus*, *Salmo*, *Salvelinus*, and *Thymallus*) and two ST8Sia IV-related sequences were identified only in the Cypriniformes *C. carpio* and *Sinocyclocheilus anhuiensis* (Supplemental Table S1). We took advantage of the improved genome and transcriptome sequencing of several fish [37,50], selected several representative Salmoniformes and Cypriniformes ST8Sia sequences, and constructed phylogenetic trees (Supplemental Figure S1). The topology of these trees indicated that the later duplications of *st8sia* genes were not associated to the Teleost specific genome duplication (TGD, WGD R3), but rather to more recent lineage-specific genome duplication events described in Salmonidae (SGD) lineage [51] and in *C. carpio* species [52].

2.3. Synteny and Paralogy Analyses of the st8sia2, st8sia4, st8sia3, and st8sia9 Gene Loci

To explain the gain or loss of ST8Sia subfamilies, we further analyzed the evolutionary relationships between these *st8sia* genes. The kind of event that created duplication was characterized by analyzing the conserved synteny between ST8Sia paralogues. It was expected that the *st8sia* genes created by a WGD would be far apart on different chromosomes in one genome, but surrounded by similar genes in each of the duplicated regions (i.e., paralogons). Significant Tetrapod paralogons containing *st8sia2* and *st8sia4* genes were found and a well conserved synteny could be established for *st8sia2* and *st8sia4* gene loci in Tetrapods (i.e., human, mouse, chicken, and xenopus) genomes (Figure 3A) as previously described [14]. However, in the fish genomes, as the *st8sia2* gene was absent in Esociformes and Siluriformes, we considered the neighboring *furin*, *fes*, *sv2b*, *fam147b*, *mctp2*, and *chd2* genes around *st8sia2* on the medaka chromosome 6 to retrieve the synteny on *Esox lucius* LG19 and on *Ictularus punctatus* chromosome 4. Similarly, *ppip5k2*, *pam*, *chd1 erap1a*, and *syk* genes conserved around the *st8sia4* locus were used to retrieve the synteny on *O. latipes* chromosome 12, *Gasterosteus oculatus* chromosome XIV, and *Xiphophorus maculatus* chromosome 8. Interestingly, paralogues of these genes could be identified on other chromosomes in the various fish genomes indicative of an ancient Teleost specific WGD (TGD) followed by intense gene rearrangements. This further suggests that the *st8sia* genes have undergone the TGD and the duplicated *st8sia* genes were rapidly lost during Teleost evolution. In the Salmoniformes, a highly conserved synteny was found around the two *st8sia2-r* gene loci corresponding to one ohnologous region in the spotted gar (*L. oculatus*), likely resulting from the fourth round of WGD (SGD) that took place more recently in the Salmoniforme genomes [51]. The two *st8sia4-r* genes were localized on two distinct chromosomes in *C. carpio* genome, supporting the hypothesis of a more recent species-specific genome duplication event in *C. carpio* [52] in spite of a weak synteny conservation (Figure 3A).

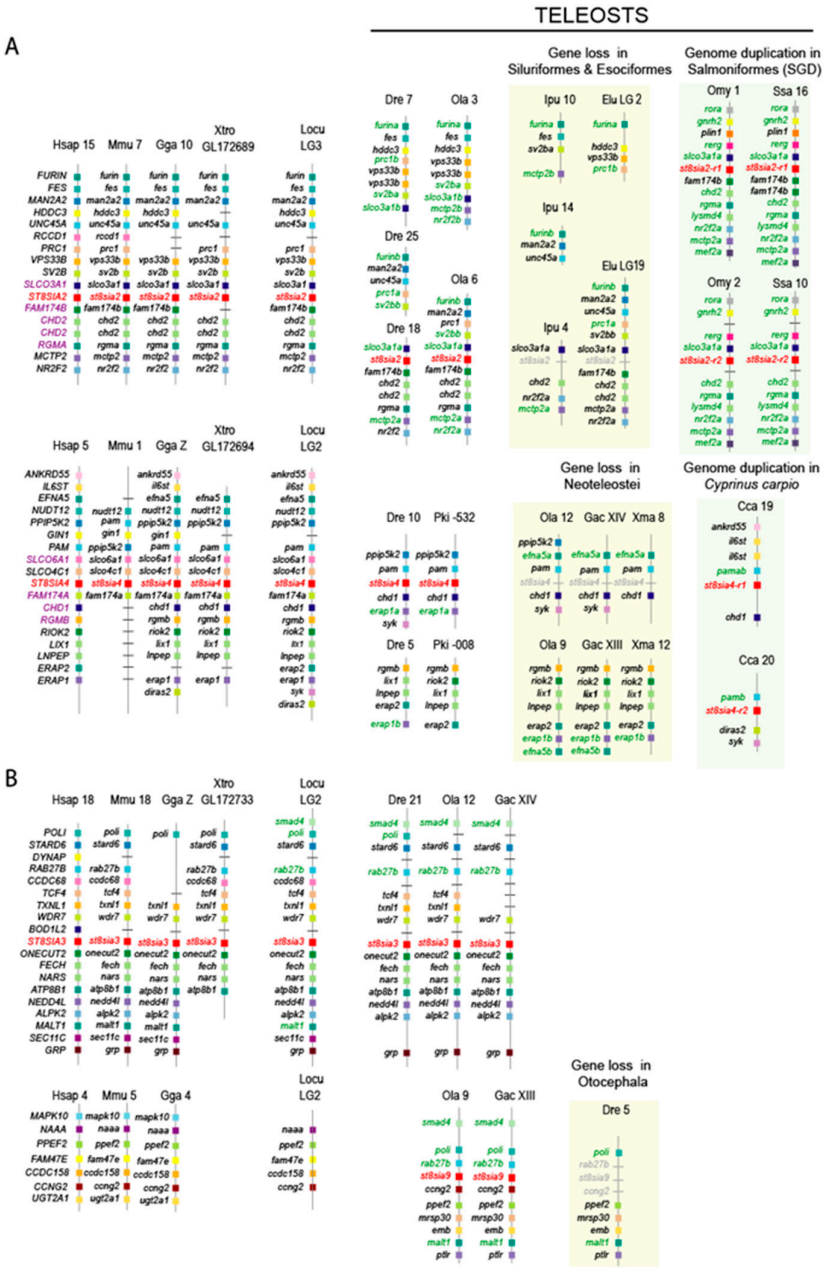

Figure 3. Syntenic relationships of the oligo- and poly-α2,8-sialyltransferases gene loci in vertebrates. Chromosomal locations of the *st8sia* genes and neighboring gene loci were determined in the human (*Homo sapiens*, Hsa), the mouse (*Mus musculus* (Mmu), the chicken (*Gallus gallus*, Gga), the spotted gar (*L. oculatus*, Locu), the western clawed frog (*Xenopus tropicalis*, Xtro), the zebrafish (*D. rerio*, Dre), the Japanese medaka (*O. latipes*, Ola), the channel catfish (*I. punctatus*, Ipu), the northern pike (*E. lucius*, Elu), the rainbow trout (*O. mykiss*, Omy), the Atlantic salmon (*Salmo salar*, Ssa), the African weakly electric fish (*Paramormyrops kingsleyae*, Pki), the three-spined stickleback (*G. aculeatus*, Gac), the southern platyfish (*X. maculatus*, Xma), and the European carp (*C. carpio*, Cca). Information from the National Center for Biotechnology Information (NCBI) and ENSEMBL release 97 was used to identify putative orthologues, which were visualized using the Genomicus 97.01 [53]. Paralogous genes in the fish genomes are indicated in green and in purple in the human genome. The *st8sia* genes are indicated in red or in grey when lost. (**A**) Syntenic relationships of the *st8sia2* and *st8sia4* gene loci in vertebrates. (**B**) Syntenic relationships of the *st8sia3* and *st8sia9* gene loci in vertebrates.

The synteny around the *st8sia3* gene locus including *wdr7*, *onecut2*, and *fech* genes is highly conserved in vertebrate lineages from fish to mammals (Figure 3B). Synteny around *st8sia9* locus is less conserved and is limited to a smaller syntenic block with *ccng2* and *ppef2* genes, which is reminiscent of ancient WGD followed by intrachromosomal rearrangement in the ancestral fish genome.

Altogether, our phylogenetic analyses enabled us to refine the evolutionary history of the fish ST8Sia and to propose a model of their evolution illustrated in Figure 4, which agrees with the fish phylogenetic tree of life [54]. It is interesting to note that, while Braasch and Postlethwait (2012) determined duplicated gene retention rates of 12–24% after the TGD 320 MYA [55], we observed no remaining *st8sia* gene copy from this event and no modification on the fish ST8Sia repertoire. However, more recent polyploidization events were recorded in several families (Salmonidae, 80 MYA), genera (Anguilla) or species (*C. carpio*, 8 MYA), which impacted the overall poly-α2,8-sialyltransferases repertoire. In Salmonidae, we described only two remaining *st8sia2* duplicates after the Ss4R among the eight ancestral *st8sia* genes (12% duplicate retention), while Lien *et al.* (2016) revealed a global retention rate around 55% [56]. In the carp *C. carpio*, two *st8sia4* genes were retained as duplicates among the seven *st8sia* genes (14% duplicate retention), while Li et al. (2015) calculated a global value of 92% [57]. Furthermore, these studies highlighted the fact that the retained genes after tetraploidization were specifically involved in signal transduction, protein complex formation, and immune system, which prompted us to focus on the functional divergence of these poly-α2,8-sialyltransferase duplicated genes (neofunctionalization) and on their expression divergence (subfunctionalization).

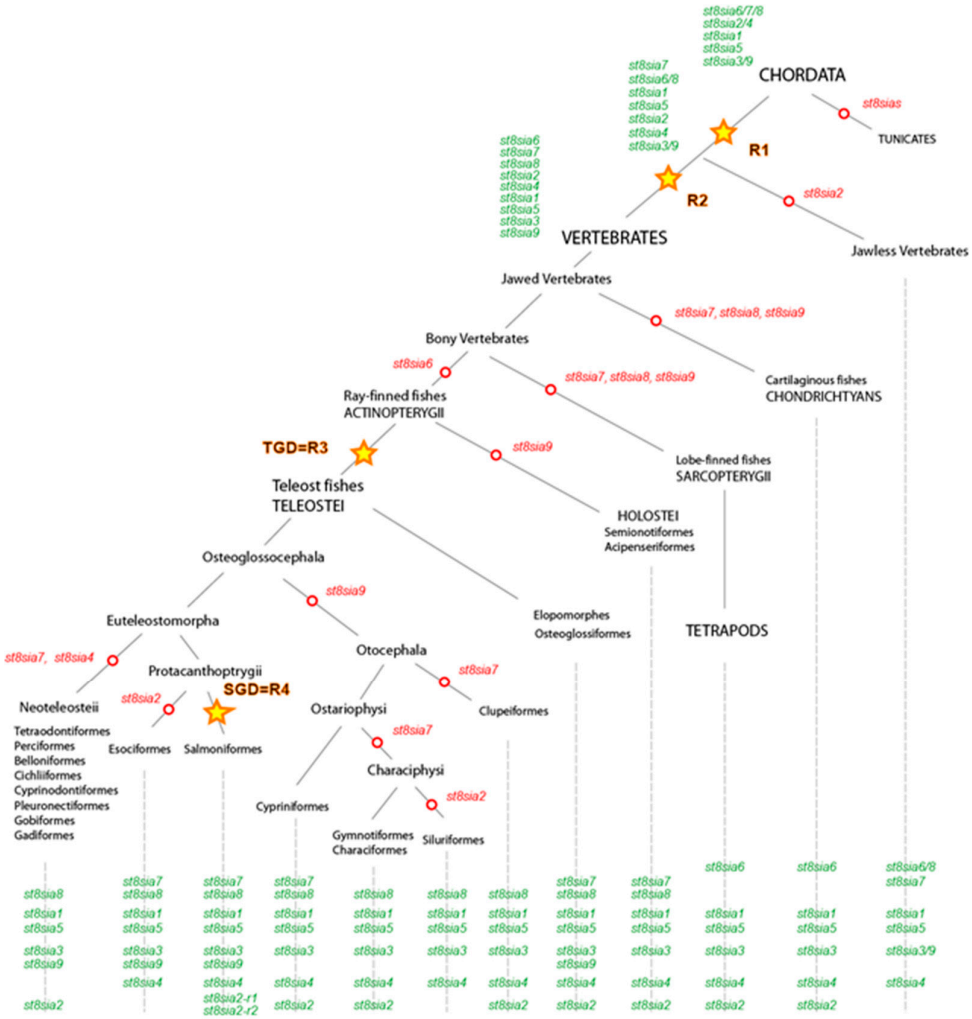

Figure 4. Schematic representation of the ST8Sia family evolution in the ray-finned fishes. This model for the evolution of *st8sia* genes is based on the evidence from protein sequence phylogeny, conserved synteny of genomic *st8sia* loci in vertebrate species and their paralogous relationships in fish genomes. The model takes into account the evolution of five ancestral groups of ST8Sia (*st8sia6*/*7*/*8*, *st8sia2*/*4*, *st8sia1*, *st8sia5*, and *st8sia3*/*9*) indicated in green and present in the ancestor of Chordates that predate the WGD R1 and WGD R2. Open red circles depict gene losses on the phylogenetic tree and yellow stars correspond to the WGDs R1, R2, R3 (teleost specific duplication, TGD), and R4 (salmonids specific duplication, SGD).

2.4. Molecular Evolution of the Poly-α2,8-Sialyltransferases

A remarkable difference between α2,8-linked polySia chains found in mammals and salmonid fish seems to be the structural diversity of polySia in fish [58–60]. Whereas in mammals, homopolymers of Neu5Ac residues are typically formed [61], in rainbow trout eggs, polymers can consist of Neu5Ac, Neu5Gc, and KDN in addition to their O-acetylated forms [62]. One explanation could be a better accessibility to different sialic acids in fish, because, in transgenic mice—showing a Neu5Gc overexpression in brain—besides Neu5Ac, Neu5Gc also seems to be utilized to build polySia [63].

Another explanation might be the occurrence of structural changes of the protein backbone during the evolution of the polysialyltransferases. We thus investigated the potential consequences of specific-lineages' *st8sia* gene loss and duplication on the functional fate of duplicates, an issue that is still poorly understood [64,65]. Substitution rate analysis of the duplicated *st8sia2* genes maintained in Salmoniformes genome after the SDG event indicated four amino acid substitutions in the ST8Sia II-r2 coding sequences compared with ST8Sia II-r1 and the rest of Teleost ST8Sia II sequences, while there were only two substitutions in the ST8Sia II-r1 sequence. Of particular note, among the four substitutions found in ST8Sia II-r2, the H → Y is recorded in sialylmotif L, and the R → Q between sialylmotifs S and III, whereas the two substitutions in the ST8Sia II-r1 sequence are located nearby the PSTD motif (Figure 5A). In addition, two convergent substitutions leading to the same amino acid were identified near the end of sialylmotif L (i.e., acquisition of a G from a Q) and beyond the sialylmotif III (i.e., acquisition of an H from an S), respectively. These drastic modifications in amino acid properties in functionally important locations in the catalytic domain of these salmonid ST8Sia II let us suggest profound changes in both ST8Sia II functions (i.e., neofunctionalization). Likewise, we examined the impact of *st8sia4* loss on the remaining *st8sia2* gene in Neoteleostei using parsimony analysis. We found two substitutions, A → S and Q → S, located in the sialylmotif L and between the sialylmotifs III and VS that of Neosteleostei ST8Sia II, respectively (Figure 5B). Interestingly, we also found a convergent T → K substitution located between the sialylmotifs III and VS that of Neosteleostei ST8Sia II that restores the K amino acid characteristic of all the ST8Sia IV sequences (Figure 5B), further suggesting changes in ST8Sia II functions in Neoteleotei. No substitution could be detected in ST8Sia IV sequences after the loss of *st8sia2* gene in Esociformes and Osmeriformes. Finally, we recorded the substitutions on the ancestral sequence of ST8Sia III after ST8Sia IX loss in Otocephala. We observed three substitutions in ST8Sia III sequence: V → T near the sialylmotif L, A → T in the sialylmotif VS, and Y → F beyond (Figure 5C).

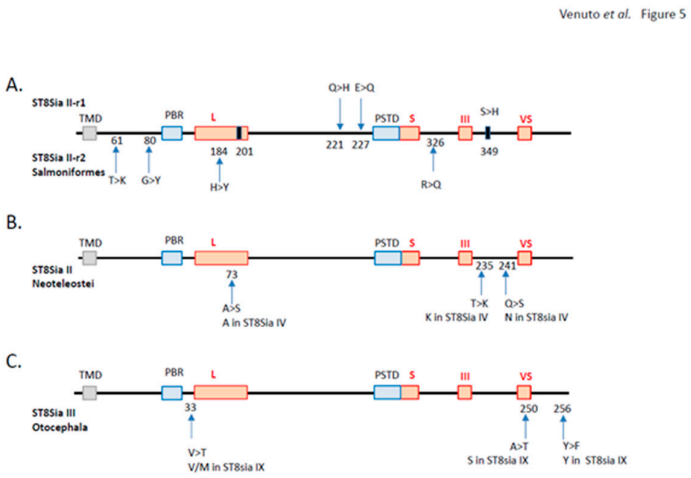

Figure 5. Substitution rate analysis of the impact of *st8sia* gene duplications and losses. The sialylmotifs are indicated by red boxes and the transmembrane domain by a grey box. (**A**) Duplication of *st8sia2* genes in Salmoniformes. The substitutions observed in ST8Sia II-r1 and ST8Sia II-r2 are indicated by an arrow above and below, respectively. The position of the substitutions corresponds to the alignment in Supplemental Data 2. The black rectangles correspond to convergent mutations retrieved in both sequences. In T > K, for example, T is the ancestral state and K is the derived one. (**B**) Impact of *st8sia4* gene loss in Neoteleostei on the remaining fish ST8Sia II sequences. The code for substitution is the same as in A. The corresponding amino acid present in the paralogue ST8Sia IV sequence is given below. (**C**) Impact of *st8sia9* gene loss in Otocephala on the remaining fish ST8Sia III sequences (same abbreviations as in B).

The most striking domain of both polysialyltransferases—ST8Sia II and ST8Sia IV—is PSTD, which is essential for the polysialylation of NCAM [31,66]. This motif contains a high number of basic amino acids and is important for substrate binding and the catalytic activity. Troy and co-workers exchanged several of these amino acids to determine their distinct impact on the enzymatic activity of human ST8Sia IV [31]. Doubled substituent mutants with an exchange of the first basic residues (declared as K2 and K4 in Figure 6) by neutral amino acids retained approximately 80% of the enzyme activity and comparable values were determined, when only K6 was replaced. Stronger effects were observed in single substituted mutants where R8, H18, K28, K32, or R33 was replaced by a neutral amino acid. All these changes reduced activity by more than 50%. Their experiments demonstrated that, in addition to the neutral amino acid I31 (mutants retained only 6% of their activity), especially the basic amino acids of PSTD were key elements for polysialylation. Most of these important amino acids of the human ST8Sia IV are also highly conserved in the fish enzyme. Changes occurred sporadically at K2, K4, K6, and R8 in individual fish species (Figure 6). On the basis of the work of Troy and co-workers [31], the R8 change may have the highest impact on the general enzyme activity, as a replacement of this amino acid reduced the activity to less than 25%. However, we observed an exchange of R8 only in three fish species including *I. punctatus*. Nevertheless, as mentioned above, other substituted amino acids may also influence the interaction with the nascent sialic acid chain, depending on the composition (Neu5Ac, Neu5Gc, KDN, and O-acetylated variations) of the polySia chain.

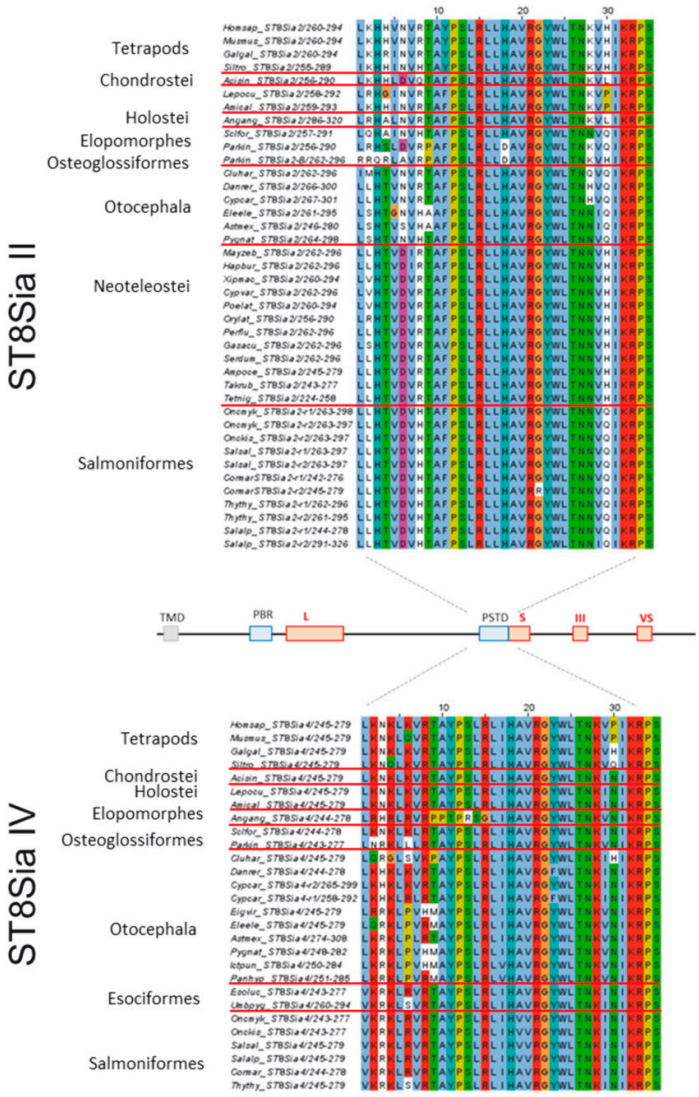

Figure 6. Sequence-based analysis of the polysialyltransferase domain (PSTD) in fish ST8Sia II and ST8Sia IV. Multiple sequence alignment of PSTD were performed with CLUSTAL OMEGA of EMBL-EBI by MUSCLE (3.8) edited and annotated in Jawa Alignment Jalview [67]. The used protein entries from different species are listed in Supplemental Table S1. The different colors from Clustal X scheme codes indicate the following characteristics: hydrophobic (blue), positive charge (red), negative charge (magenta), polar (green), cysteine (pink), glycine (orange), proline (yellow), aromatic (cyan), and gap (white). It should be noted that one additional amino acid was added to the N-terminus and two additional amino acids to the C-terminus of PSTD.

More consistent variations were observed when ST8Sia II sequences were compared. In addition to the mentioned K2 and H4 (K instead of H in ST8Sia IV), an exchange of a basic amino acid occurs more frequently and is often highly conserved within one family. For instance, in Salmoniformes, lysine residues at position 2 and 28 are changed with apolar amino acids and the strongly basic R8 residue is exchanged with histidine, which is only partly positively charged at neutral pH. On the basis of the studies of Nakata et al. using human ST8Sia IV, we can also assume remarkable changes in the enzymatic activity of ST8Sia II [31]. For instance, ST8Sia IV mutants with a neutral amino acid at position K28 retained less than 25% of their enzymatic activity. This is in line with studies by Kitajima and co-workers demonstrating that rainbow trout ST8Sia II isoforms show only low enzymatic activity in vitro [33]. Intriguingly, in Neoteleostei, the very important lysine at position 28 was also exchanged with a neutral amino acid. Notably, in contrast to Salmoniformes, in

Neoteleostei, ST8Sia II is the only polysialyltransferase because ST8Sia IV is absent. The presence of only one polysialyltransferase in Neoteleostei, which additionally includes such a striking mutation, suggests that polysialylation significantly changed in Neoteleostei in comparison with other vertebrates.

In addition to sequence alignments, we simulated the PSTD 3D structure of fish ST8Sia II and ST8Sia IV, based on the determined 3D structure of human ST8Sia IV PSTD (PDB 6AHZ) (Figure 7), which were published by Peng and colleagues [66]. Volkers et al. described that PSTD acts as a basic furrow, leading the nascent sialic acid chain to the active site of the polysialyltransferase [32]. The 3D simulation of the human ST8Sia IV PSTD shows that only significant differences between the electrostatic potential surfaces are detectable at the N-terminal region. Especially the orientation of the basic areas changed between the species. In contrast, the central and C-terminal area exhibited only minor changes. In the case of ST8Sia II, the most prominent alterations also occurred at the N-terminal domain (Figure 8). However, exchanging the N6 with aspartate, an exposed acidic segment is formed in Salmoniformes and Neoteleostei, which may influence the interaction between PSTD and the negatively charged sialic acid polymers. However, regarding the 3D simulation of PSTD, it has to be noted that a simulation is only a simulation and crystal structures of PSTD in addition to the whole enzymes are necessary for the generation of unambiguous 3D models.

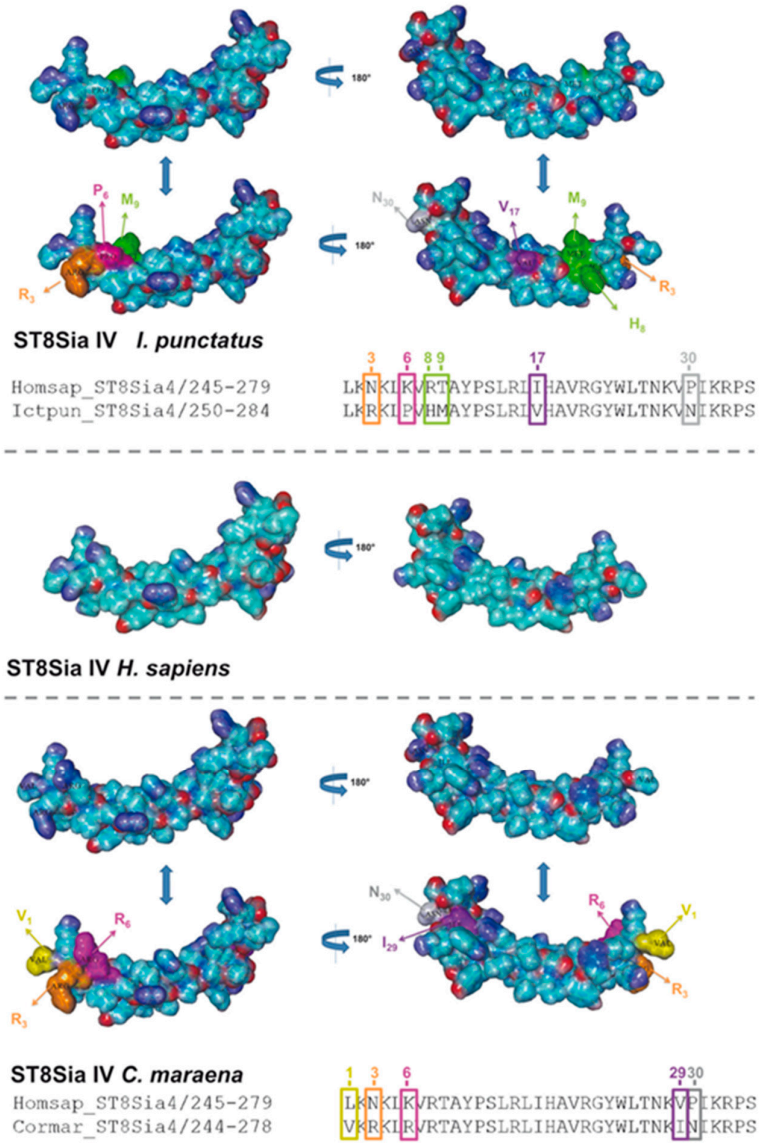

Figure 7. Three-dimensional (3D) structure of PSTD motifs in fish ST8Sia IV. The 3D model of human ST8Sia IV PSTD (Protein Data Bank entry 6AHZ)—electrostatic potential surfaces—is displayed in addition to the simulated structure of PSTD from I. punctatus and *C. maraena* using YASARA. The exchanged amino acids are colored in an additional version of the 3D structure to highlight the position of the exchange: N3 → R3 (orange), K6 → P6 (magenta), R8 → H8 (green), T9 → M9 (green), I17 → V17 (violet), and P30 → N30 (grey) for *I. punctatus* and L1 → V1 (yellow), N3 → R3 (orange), K6 → R6 (magenta), V29 → I29, and P30 → N30 (grey) for *C. maraena*. It should be noted that, for the determination of the 3D structure of human ST8Sia IV PSTD, a peptide was used with one additional amino acid on the N-terminus and two additional amino acids on the C-terminus of PSTD [66].

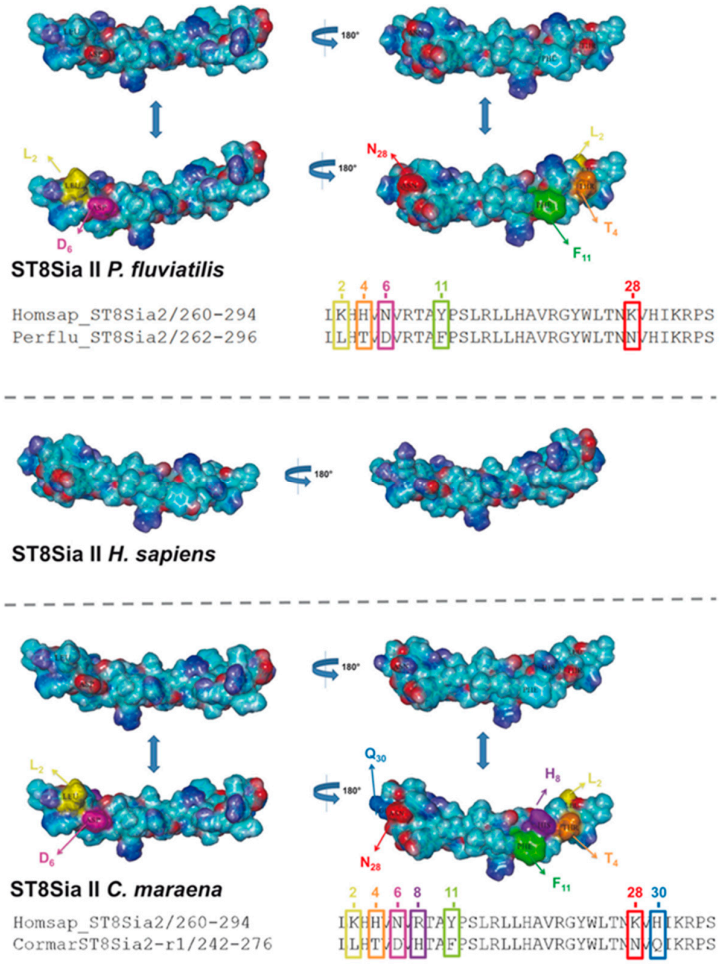

Figure 8. Three-dimensional (3D) structure of PSTD motifs in fish ST8Sia II. The 3D model of human ST8Sia II PSTD in addition to PSTD from *P. fluviatilis* and *C. maraena* was simulated, based on the 3D model of human ST8Sia IV PSTD (Protein Data Bank entry 6AHZ) using YASARA. The electrostatic potential surfaces are displayed. The exchanged amino acids are colored in an additional version of the 3D structure to highlight the position of the exchange: K2 → L2 (yellow), H4 → T4 (orange), N6 → D6 (magenta), Y11 → F11 (green), and K28 → N28 (red) for *P. fluviatilis* and K2 → L2 (yellow), H4 → T4 (orange), N6 → D6 (magenta), R8 → H8 (violet), Y11 → F11 (green), K28 → N28 (red), and H30 → Q30 (light blue) for *C. maraena*. For the determination of the 3D structure of human ST8Sia IV PSTD, a peptide with one additional amino acid on the N-terminus and two additional amino acids on the C-terminus of PSTD were used [66].

Taken together, our sequence alignments and 3D simulations demonstrate that, in fish, characteristic alterations of the amino acid sequences occurred within PSTD and that several of these

replaced amino acids are important for the enzymatic activity in the case of human ST8Sia IV, as demonstrated by Troy and co-workers [31]. These variations might also influence the ability of PSTD to interact with sialic acid chains consisting of other sialic acids than Neu5Ac, such as Neu5Gc and KDN, as well as their O-acetylated forms. However, to definitively proof this hypothesis of neofunctionalization of fish polysialyltransferases, their enzymatic activity has to be characterized in more detail.

2.5. Expression of Polyα2,8-Sialyltransferase Genes in C. Maraena *Tissues*

Having characterized the chromosomal localization, evolutionary history, and structure of the poly-α2,8-sialyltransferases ST8Sia II and ST8Sia IV encoded by the *st8sia2* and *st8sia4* genes, respectively, we eventually profiled their expression in ten organs and tissues of *C. maraena* as a representative of the Salmoniformes (Figure 9A,B). As *st8sia2* is duplicated in salmonid fishes, we investigated whether the expression of both genes is tissue-specific, and thus possibly function-specific. To this end, discriminating primer pairs for *st8sia2-r1* and *st8sia2-r2* as well as for *st8sia4* transcripts were designed. The RT-qPCR analysis revealed that *st8sia2-r1* transcripts were on low levels in liver, heart, spleen, head kidney, gills, hypothalamus, and hind brain (>300 copies/ng RNA), and almost absent in muscle (>10 copies/ng RNA) (Figure 9A). In stark contrast, the copy numbers of *st8sia2-r1* were at a high level in gonads (~1700 copies/ng RNA) and telencephalon (~ 2140 copies/ng RNA) (Figure 9B). The transcript levels of the gene copy *st8sia2-r2* were generally higher compared with its paralogue, ranging from a 1.5-fold difference in gonads to a 233-fold difference in spleen (Figure 9A). While the expression of *st8sia2-r2* was not detectable in hind brain and telencephalon, it exceeded the expression level of *st8sia2-r1* by 4622-fold in the hypothalamus.

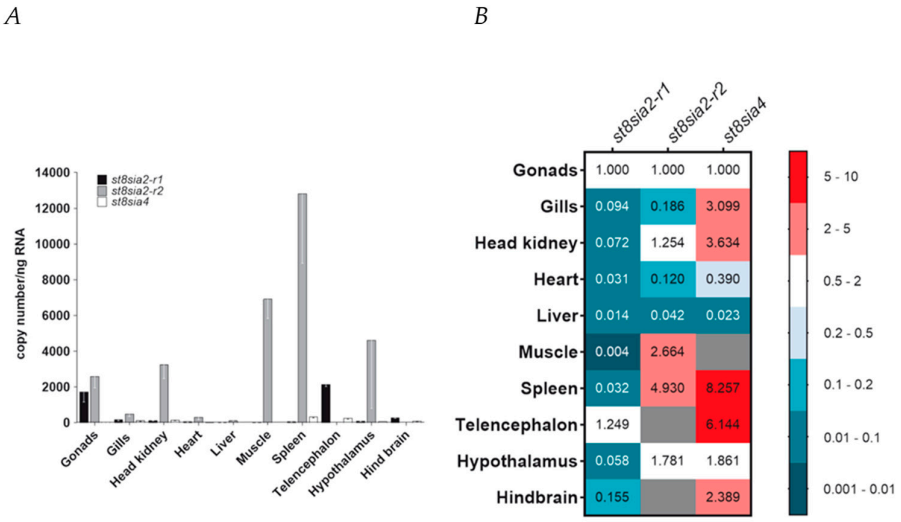

Figure 9. Expression profiling of poly-α2,8-sialyltransferase-encoding genes in maraena whitefish. (**A**) Transcript levels of *st8sia2-r1* (black bars), *st8sia2-r2* (gray), and *st8sia4* (blank) were determined in ten tissues from maraena whitefish (n = 4), as indicated on the abscissa. Bars represent the averaged copy numbers normalized against three reference genes; error bars represent the standard deviation. (**B**) A heat map represents the same copy numbers per target gene as shown in (A) relative to the expression in gonads (set as 1.0). These relative expression values are colored according to the code given at the right. Non-detectable transcript numbers are indicated by gray fields.

The expression level of *st8sia4* was at a similarly low or even significantly lower level compared with that of *st8sia2-r1* with the highest copy numbers in spleen (~330 copies/ng RNA). No or only very few *st8sia4* transcripts were detectable in liver, muscle, and heart (Figure 9B). The results are partially different in comparison with the determined mRNA levels in rainbow trout using Northern blot analysis and semi-quantitative PCR [33]. For instance, spleen samples were negative for *st8sia2*

transcripts, which might not only be the result of differences in the applied methods, but also in general differences between these two Salmoniformes.

Taken together, profiling the expression of the poly-α2,8-sialyltransferase genes revealed a tissue-specific expression pattern of *st8sia2* genes in *C. maraena* tissues indicative of their subfunctionalization. Probably one of the most striking differences between the expression profiles in maraena whitefish and humans is the presence of *st8sia2* and *st8sia4* transcripts in the reproductive tract. Whereas in humans, only a weak signal for *st8sia2* mRNA and no signal for *st8sia4* mRNA could be detected by Northern blotting [68], in *C. maraena,* the gonads belongs to the tissues with the highest expression levels of polysialyltransferases. This was already described by Kitajima and colleagues using rainbow trout ovaries [33]. Besides the gonads, remarkable differences were also observed in spleen. Contrary to humans, where no *st8sia2* mRNA was detectable [68], *st8sia2-r2* expression was extremely high in the spleen of *C. maraena*, indicating that ST8Sia II-r2 might play a role during immunologic reactions in maraena whitefish. Altogether, these results let us suggest that, in addition to the number of active polysialyltransferases, as well as their enzymatic activity, the physiological roles of these polysialyltransferases may have changed during the evolution of vertebrates.

3. Materials and Methods

3.1. Materials and Animals

Maraena whitefish were provided by the Institute for Fisheries of the State Research Centre for Agriculture and Fishery Mecklenburg-Western Pomerania (Born, Germany), and BiMES, Binnenfischerei GmbH (Friedrichsruhe, Germany). Fish were held in fresh-water recirculation systems with a 12:12 day-and-night cycle at 18 °C. Water quality was maintained by automated purification and disinfection (bio-filter and UV light). In addition, the concentrations of selected chemical and physical water parameters were constantly determined.

Sampling of ten organs or tissues (gills; gonads; head kidney; heart; liver; muscle; spleen; and the brain regions hypothalamus, hind brain, and telencephalon) from four maraena whitefish followed the standards described in the German Animal Welfare and was approved by the Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei, Mecklenburg-Vorpommern, Germany (LALLF M-V/TSD/7221.3-1-069/18) in November 2018. The tissues were sampled rapidly and immediately frozen in liquid nitrogen to be kept at −80 °C until RNA extraction.

3.2. In Silico Identification and Phylogenetic Analysis of ST8Sia Sequences

A local alignment BLAST approach was used to retrieve the vertebrate *st8sia* nucleotide sequences with significant homology to the mammalian sequences from the genomic and Transcriptome Shotgun Assembly (TSA) divisions of the GenBank/EBI databases at the National Center for Biotechnology Information (NCBI) (last accessed on 27 September 2019), ENSEMBL (release 97) and from the PhyloFish database [7,14,37]. The protein sequence analysis was performed using the Expert Protein Analysis System (ExPASy; Swiss Institute of Bioinformatics, Switzerland; website (https://www.expasy.org/)). Sequence alignments were performed using the clustalW (PRABI; https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_clustalw.html). Phylogeny was determined aligning the known vertebrate ST8Sia sequences with MUSCLE in MEGA7.0 [40]. The multiple sequence alignments of the selected vertebrate ST8Sia sequences were conducted using MUSCLE and Clustal Omega algorithms in MEGA7.0 and manually refined (see Supplementary Data 1 and 2). Phylogenetic trees were produced by the neighbor-joining (NJ), maximum likelihood, and minimum evolution method in MEGA 7.0 [40,69].

To determine the consequences of duplication or loss of genes of a given order of Actinopterygian, we considered what happened on its closest paralogue. The amino acid substitutions that occurred at its base were deduced using the parsimony method implemented in Protpars program (PHYLIP package vers. 3.69) [70].

3.3. Synteny Analysis, Paralogon Detection, and Ancestral Genome Reconstruction

Synteny between the *st8sia* gene loci and neighbour genes in vertebrate genomes was assessed by manual chromosome walking and reciprocal BLAST. Detection of paralogous blocks was visualized with Genomicus (version 97.01) http://www.genomicus.biologie.ens.fr/genomicus-92.01/cgi-bin/search.pl, last accessed August 2019 [53]. When the *st8sia* gene of interest was not found in a genome, physically close genes were used as a seed to identify syntenic segments.

3.4. Sequence Alignments, Motifs Analysis, and 3D Simulation of PSTD

Multiple sequence alignments were performed with MUSCLE of EMBL-EBI (version 3.8; https://www.ebi.ac.uk/Tools/msa/muscle/) from selected species using published sequences (accession numbers in Supplemental Data 1). The sequences were conducted, edited, and annotated in Jawa Alignment Viewer Jalview 2.11.0, and manually refined [67]. The 3D structure of the human PSTD of ST8Sia II as well as ST8Sia IV was generated in YASARA (Version 19.9.17) using the following Protein Data Bank (PDB) entries: ST8Sia IV (code: 6AHZ) (PDB, https://www.rcsb.org/pdb/home/sitemap.do https://www.wwpdb.org/pdb?id=pdb_00006ahz). The ST8Sia II was generated in YASARA changing the amino acid in the positions N3, K4, L5, and K6, corresponding to H3, H4, V5, and N6, respectively. The human amino acid sequences of ST8Sia II and ST8Sia IV were modified at the positions with the following amino acids according to the different fish PSTD sequences: *C. maraena* ST8Sia II, L2, T4, D6, H8, F11, N28, and Q30; *P. fluviatilis* ST8Sia II, L2, T4, D6, F11, and N28; I. punctatus ST8Sia IV, R3, P6, H8, M9, V17, and N30; and *C. maraena* ST8Sia IV, V1, R3, R6, I29, and N30. We designed only one PSTD motif of *C. maraena* because there is only one difference at position R22 between ST8Sia II-r1 and ST8Sia II- r2.

3.5. RNA Extraction, cDNA Synthesis, Primer Design, and RT-qPCR

Total RNA was isolated from the individually homogenized organs and tissues using TRIzol (Invitrogen/Thermo Fisher Scientific, Darmstadt Germany), followed by an additional purification step (RNeasy Mini Kit, Qiagen). The quantity and integrity of the isolated RNA were determined using the NanoDrop 2000 photometer (Thermo Fisher Scientific) and agarose gel electrophoresis. Subsequently, we reverse-transcribed the total RNA using the SuperScript II Reverse Transcriptase (Thermo Fisher Scientific) and a mixture of oligo-d(T) and random hexanucleotides. This reaction was carried out at 42 °C (50 min), followed by an inactivation step (70 °C, 15 min). The resulting cDNA was diluted in 100 μL distilled water.

Real-time fluorescence-based quantitative RT-PCR (RT-qPCR) was used to determine the mRNA abundance of the two *st8sia2* gene variants in the above ten organs and tissues of maraena whitefish (n = 4). To this end, we identified discriminating sequence motifs to derive the oligonucleotides for *st8sia2-r1* (sense, 5′-AGCCTCATCAGGAAGAACATCC-3′; antisense, 5′-TTCCCTACGATGGCACAGCGT-3′) and *st8sia2-r2* (sense, 5′-CGTTCAACAGGAGCCTCTCTAA-3′; antisense, 5′-TTCCCTACGATGGCACAGCGC-3′). Moreover, we designed a *st8sia4*-specific primer pair (sense, 5′-ATGATAAGGAAGGACGTGCTGC-3′; antisense, 5′-TGTTGAGCGTTCGGCGTCTGT-3′). These RT-qPCR primers were designed (Pyrosequencing Assay Design software v.1.0.6; Biotage, Uppsala, Sweden) to synthesize amplicons between 121 bp and 226 bp. *eef1a1a2* (encoding eukaryotic translation elongation factor, variant a2), *rpl9*, and *rpl32* (ribosomal proteins L9 and L32) were selected as reference genes [71]. The RT-qPCR analyses were conducted with the LightCycler 96 System (Roche, Mannheim, Germany) using the SensiFAST SYBR No-ROX Kit (Bioline, Luckenwalde, Germany). We only considered crossing point (CP) values >35 for The expression analysis of the *st8sia2-r1 st8sia2-r2*, and *st8sia4*. The calculation of their copy numbers was based on standard curves having been generated on 10-fold dilutions of the respective PCR-generated fragments (1×10^3 to 1×10^6 copies). Melting-curve analyses validated the amplification of the

distinct products. Amplicons were visualized on 3% agarose gels in order to assess product size and quality.

3.6. Data Availability

To identify the maraena whitefish ST8Sia II sequences, the orthologous sequences from rainbow trout and Atlantic salmon were aligned with the software Bowtie2 (v 2.2.4) to our RNA-seq read collection from maraena whitefish [72]. The alignments were then indexed and sorted with the software package Samtools (v.16) and the final consensus sequences were obtained with the Ugene software (v 1.29).

4. Conclusions

In this study, we highlighted an expansion and particular distribution of the ray-finned fish ST8Sia repertoire owing to several duplications and loss events of *st8sia* genes, and we refined their evolutionary history. Our analyses of the molecular evolution in ST8Sia sequences and in key functional motifs (i.e., motif L and PSTD) let us suggest that the polysialyltransferases might evolved new enzymatic activities and/or specificities in the course of Vertebrate evolution. Their expression profiles in Salmonid tissues differ from those observed in mammals and further point to a subfunctionalization of these poly-α2,8-sialylatransferases. Altogether, we have laid the foundation for further studies towards understanding of the remarkable differences between α2,8-linked polySia chains found in mammals and fish.

Supplementary Materials: Supplementary materials can be found at www.mdpi.com/xxx/s1.

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Abbreviations

DP	degree of polymerization
Neu5Ac	N-acetylneuraminic acid
Neu5Gc	N-glycolylneuraminic
oligoSia	oligosialic acid
polySia or PSA	polysialic acid
PDB	Protein Data Bank
PSGP	salmonid egg polysialoglycoprotein
ST8Sia	α2,8-sialyltransferase
WGD	whole genome duplication
KDN	2-keto-3-deoxynononic acid
MSA	multiple sequence alignment

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5.2 Study II: Characterization of the Polysialylation Status in Ovaries of the Salmonid Fish Coregonus maraena and the Percid Fish Sander lucioperca

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Brief summary:

In vertebrates, Sia polymers, consisting of α2,8-linked Neu5Ac residues are especially important for the development of the brain. However, in contrast to the nervous system, little is known about the distribution and role of polySia in fish ovaries. The aim of this study was the characterization of the polysialylation on the fish reproductive tract. We investigated the distribution of α2,8-linked Neu5Ac polymers in ovaries of C. maraena representing a salmonid farm fish species from the Baltic region. When ovary tissue lysates were investigated by WB against α2,8-linked Neu5Ac polymers, a typical broad band is detectable. This immunostaining was abolished by a pre-treatment with endoN, which is reported to specifically degrade polySia. Using immunohistological analysis of ovaries' seriales sections, we saw that selected cell populations are polySia-positive in the early and intermediate pre-vitellogenic stage.

Due to the study, we learned that during the duplication of genome, the polySTs were lost or duplicated. Salmonidae have two st8sia2 and Percidae have no st8sia4.

Interestingly, the same cell populations were polySia-positive in ovaries of pikeperch, which is a percid aquaculture fish. Since a co-localization of polySia and the germ cell marker DDX4 was observed via immunostaining, it seems likely that oogonia are polySia positive.

cells

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Characterization of the Polysialylation Status in Ovaries of the Salmonid Fish *Coregonus maraena* and the Percid Fish *Sander lucioperca*

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Abstract: In vertebrates, the carbohydrate polymer polysialic acid (polySia) is especially well known for its essential role during neuronal development, regulating the migration and proliferation of neural precursor cells, for instance. Nevertheless, sialic acid polymers seem to be regulatory elements in other physiological systems, such as the reproductive tract. Interestingly, trout fish eggs have polySia, but we know little of its cellular distribution and role during oogenesis. Therefore, we localized α2,8-linked *N*-acetylneuraminic acid polymers in the ovaries of *Coregonus maraena* by immunohistochemistry and found that prevalent clusters of oogonia showed polySia signals on their surfaces. Remarkably, the genome of this salmonid fish contains two *st8sia2* genes and one *st8sia4* gene, that is, three polysialyltransferases. The expression analysis revealed that for *st8sia2-r2*, 60 times more mRNA was present than *st8sia2-r1* and *st8sia4*. To compare polysialylation status regarding various polySiaT configurations, we performed a comparable analysis in *Sander lucioperca*. The genome of this perciform fish contains only one *st8sia2* and no *st8sia4* gene. Here, too, clusters of oogonia showed polysialylated cell surfaces, and we detected high mRNA values for *st8sia2*. These results suggest that in teleosts, polySia is involved in the cellular processes of oogonia during oogenesis.

Keywords: sialic acid; polysialic acid; sialyltransferases; Salmonidae; Percidae; oogenesis; previtellogenesis stages; primordial germ cells; oogonia

1. Introduction

Sialic acids, a heterogeneous family of acidic monosaccharides, are ubiquitous on the surfaces of eukaryotic cells and are involved in many crucial cellular events [1]. Besides the typical terminal monosialyl residues, sialic acid polymers can also be synthesized in vertebrates. However, in comparison to monosialylation, elongation by further sialic acid residues is mostly restricted to selected cell types and/or distinct points of differentiation [2–4]. For instance, proliferating populations of smooth muscle cells are polysialic acid (polySia)-positive during the postnatal development of murine epididymis, whereas at postnatal day 25, no significant polysialylation occurs in these contractile cell clusters [5].

In mammals, sialic acids polymers contain only α2,8-linked *N*-acetylneuraminic acid (Neu5Ac) residues; so far, no polymers with other sialic acids or types of glycosidic bonds (e.g., α2,9-linkages)

have been found in vivo. In contrast, fish eggs can contain a high variety of different sialic acid polymers. Inoue and colleagues observed that fertilized and unfertilized eggs from Salmonidae contained sialic acid polymers [6–8]. Interestingly, in addition to Neu5Ac homopolymers, they found more α2,8-linked polySia species, such as *N*-glycolylneuraminic acid (Neu5Gc) homopolymers, as well as hybrid structures of different sialic acid species that could also be *O*-acetylated [9,10]. The main protein carrier of these polySia chains seems to be *O*-glycans of the polysialoglycoprotein (PSGP) [8,11].

The sialyltransferases, which catalyze the biosynthesis of α2,8-linked polySia, belong to the α2,8-sialyltransferase (*st8sia*) gene family [12,13]. In mammals, two polysialyltransferases (polySiaTs) are known: ST8SiaII and ST8SiaIV. However, in ray-finned fish, poly-α2,8-sialyltransferase has a particular distribution as a result of several whole-genome duplication (WGD) and gene loss events [14]. For instance, in the salmonid genomes, in addition to *st8sia4*, two *st8sia2* gene loci have been described [15]. Comparatively, in percid genomes, a loss of *st8sia4* took place during evolution, so only one *st8sia2* gene now exists [12,16].

Whereas the presence of polysialyltransferase genes in various fish lineages [16] and the chemical composition of polySia on maturated trout eggs are well known [9,11,17–19], only limited knowledge of the occurrence and role of α2,8-linked Neu5Ac polymers during oogenesis is available for fish. For this reason, we analyzed the polysialylation status of the ovaries of maraena whitefish *(Coregonus maraena; C. maraena*), a teleost fish with economic relevance in the Baltic area, which belongs to the salmonid family of bony fishes [20]. The histological distribution of α2,8-linked Neu5Ac polymers in the ovaries of *C. maraena* was compared with the polysialylation status of *Sander lucioperca* (*S. lucioperca*), since the genome of this percid fish contains one *st8sia2* and no *st8sia4* gene, whereas the genome of the salmonid fish *C. maraena* contains two *st8sia2* genes and one *st8sia4* gene [12,16]. Thus, a comparison of their polysialylation statuses is possible regarding different polySiaT configurations.

2. Materials and Methods

2.1. Sample Collection

Female samples from *C. maraena* were provided by the Institute of Fisheries of the Mecklenburg-Vorpommern Research Center for Agriculture and Fisheries (Born, Germany). Maraena whitefish were raised in a recirculation aquaculture system (RAS), water temperature between 20 and 22 °C, maintained by an automated purification and disinfection system with a 16:8-h day-night cycle. From August to November, water temperature was reduced during 74 days to 4 °C and fish were kept at that temperature for out-of-season reproduction. Day-night light cycles were adapted during that period according to natural conditions. Fish were fed with commercial pellets feed. The fish were caught monthly using a sieve with a net. Specimens were sacrificed following the standards described in the German Regulations for Animal Protection (2006) (TierSChG) and current German Regulations for Animal Protection and Slaughter as of 20 December 2012 (TierSchlV). Ovarian tissues from each fish were used for paraffin histology, and the remaining tissue was snap frozen at −80 °C for future protein extraction and RNA isolation.

Ovarian *S. lucioperca* samples, used for immunohistochemistry, were provided by Fischerei Loch (Hohen Sprenz, Germany). The ovaries were collected from animals processed for food production. The animals are from cage systems, which are placed in the lake Hohen Sprenz.

For mRNA analysis, *S. lucioperca* were provided by the Institute for Fishery of the State Research Center for Agriculture and Fisheries Mecklenburg-Western Pomerania (Hohen Wangelin, Germany) and maintained in the RAS facilities of the Leibniz institute for farm animal biology (FBN). The temperature of the water was set at 22 °C, with a 12:12-h day-night cycle. Water quality was regularly monitored and maintained by automated purification and disinfection (bio-filter and UV light).

2.2. Western Blotting

Proteins from the ovaries of *C. maraena* were extracted with a TriPrep kit following the manufacturer's instructions (Nucleospin, Macherey-Nagel, Düren, Germany). The resulting samples

were resolved in 1 × RIPA Buffer, and one aliquot of each sample was treated with endoneuraminidase (endoN) (6.7 µg/mL) for 1 h at 37 °C to degrade α2,8-linked Neu5Ac polymers. In addition, a color prestained protein standard was used (broad range 10–250 kDa; Cell signaling). The samples were subjected to 7% SDS/PAGE and subsequently transferred to a PVDF membrane. The membrane was blocked with 0.5% no-fat dry milk in TBS 1× buffer. Immunostaining against polySia was done with monoclonal antibody (mAb) 735 (1 µg/mL) in 5% bovine serum albumin (BSA), which recognizes α2,8-linked Neu5Ac polymers with a degree of polymerization (DP) ≥ 8. Martina Mühlenhoff (MHH Hannover, Germany) provided mAb 735 and endoN [21,22]. Horseradish peroxidase (HRP)-conjugated secondary antibodies (donkey anti-mouse, Dianova, Hamburg, Germany) were applied for visualization by chemiluminescence signal using ECL Prime. Subsequently, a Coomassie staining of the PVDF membrane was performed (Coomassie Brilliant Blue R Dye, Merck-Millipore, Darmstadt, Germany) (staining solution: 0.1% (*w/v*) Coomassie blue in 50% (*v/v*) methanol, 7% (*v/v*) acetic acid) to control the protein transfer (loading control), since Coomassie staining is compatible with immunoblotting [23]. Pictures of the chemiluminescence signal and the Coomassie staining were taken with a Bio-Rad imaging system (ChemiDoc^{TM} MP, Feldkirchen, Germany).

2.3. Real-Time Quantitative PCR (RT-qPCR)

The total RNA from *C. maraena* ovaries was purified with the TriPrep kit (Nucleospin, Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. *S. lucioperca* ovaries were homogenized using 2.8 mm ceramic beads (Precellys, VWR/Avantor) at 6000 rpm for 30 s using the Precellys 24 Homogenizer (VWR/Avantor). The whole RNA was extracted using TRIzol Reagent (Life Technologies, Carlsbad, CA, USA) and subsequently purified with the RNeasy Mini Kit (Qiagen, Hilden, Germany). The quantity of RNA was measured with a NanoDrop One^{c} photomometer (Thermo Fisher Scientific, Darmstadt, Germany). In addition, the RNA integrity was verified by electrophoresis on 1%-agarose gels. RNA was reverse transcribed to cDNA using the SensiFAST cDNA Synthesis Kit (Bioline, London, UK) following the manufacturer's protocols.

Real-time quantitative PCR (RT-qPCR) was used to determine the expression of the polySiaTs: the two duplicated genes *st8sia2 r-1*, *st8sia2 r-2*, and *st8sia4* for *C. maraena* and *st8sia2* for *S. lucioperca*. For this purpose, the gene-specific primers were designed using software (Pyrosequencing Assay Design software v.1.0.6; Biotage, Uppsala, Sweden) to amplify the fragments between 121 and 226 bp. To identify the *st8sia2* sequences in maraena whitefish, the orthologous sequences from rainbow trout *Oncorhynchus mykiss* (*O. mykiss*) were blasted against the transcriptome of maraena whitefish [20], deposited at the NCBI Sequence Read Archive (accession code: SRP066290; BioProject PRJNA302355).

The following primers were used for *C. maraena*:

st8sia2 r-1:	5´AGCCTCATCAGGAAGAACATCC-3´ (sense)
	5´-TTCCCTACGATGGCACAGCGT-3´ (antisense)
st8sia2 r-2:	5´-CGTTCAACAGGAGCCTCTCTAA-3´ (sense)
	5´-TTCCCTACGATGGCACAGCGC-3´ (antisense)
st8sia4:	5´-ATGATAAGGAAGGACGTGCTGC-3´ (sense)
	5´-TGTTGAGCGTTCGGCGTCTGT-3´ (antisense)

The *st8sia2* sequences in pikeperch were obtained from the recently published *S. lucioperca* genome [24]. For *st8sia2* in *S. lucioperca*, the following primers were designed and applied:

st8sia2:	5´-GAGGAAGAAACTGCAAATACTGG-3´ (sense)
	5´-AGTTGTTTGACGAGAGCTTGACA-3´ (antisense)

RT-qPCR was run with the LightCycler 96 System (Roche, Mannheim, Germany) in a 12-µL reaction volume containing 16.6 ng/5 µL cDNA for *C. maraena* and 50 ng/5 µL cDNA for *S. lucioperca*, 1 µL of sense and antisense primers each, and 6 µL of SensiFast SYBR No-ROX Mix (Bioline). The RT-qPCR program comprised a denaturation phase (95 °C, 5 min) followed by 40 cycles of denaturation (95 °C,

5 s), annealing (60 °C, 15 s), elongation (72 °C, 15 s), and fluorescence measurement (72 °C, 10 s). PCR products were separated on 3% agarose gels and documented by the iBright FL1000 imaging system (Invitrogen/Thermo Fisher Scientific, Darmstadt, Germany). In addition, the specific melting curve of each target gene was evaluated to check for unspecific products. The obtained real-time data were normalized against a factor based on the geometric mean values from three suitable reference genes encoding the eukaryotic elongation factor 1-alpha 1 (gene *eef1a1*) and two ribosomal protein units (genes *rpl9* and *rpl32* for *C. maraena* or *rpl32 and rps5* for *S. lucioperca*) [25].

2.4. Histological Sample Preparation

For histology, tissue samples were cooled on ice and fixed in Bouin (picric acid, formaldehyde, and acetic acid, 15/5/1, *v/v/v*) for 24 h. In parallel, samples were fixed for 48 h using a Bouin solution with 2% sucrose. Thereafter, the samples were successively dehydrated with a stepwise alcohol gradient and cleared in xylene followed by isopropanol for 27 h in an automatic tissue processor (MT, SLEE, Mainz, Germany). All the samples were embedded after dehydration in a paraffin station (MPS/P2, SLEE, Mainz, Germany). Paraffin sections (5 µm) were cut with a microtome (LEICA RM 2165, Wetzlar, Germany) and transferred to glass slides. After deparaffinization (1 h at 60 °C, rehydrated in different alcohol steps and pressure-cooked in Tris buffer pH 9 for 3 min), the tissues were stained with hematoxylin and eosin (HE). The stained tissue sections were examined with a laser scanning microscope (Carl Zeiss Axio Observer.Z1, Oberkochen, Germany)

2.5. Immunohistochemistry Procedure

For immunohistochemistry, the deparaffinized sections were washed with PBS containing 0.2% (*w/v*) immunoglobulin G (IgG)-free BSA (Carl Roth, Karlsruhe, Germany). After three washes, tissue sections were blocked with PBS containing 2% BSA (*w/v*) for 1 h at 37 °C. To immunolocalize α2,8-linked polySia, mAb 735 (0.4 µg/mL) was used. To confirm the specific binding of mAb 735, we used two control strategies: (1) omitting the first antibody and (2) degrading the α2,8-linked polySia with endoN overnight at 37 °C (1.34 µg/mL). The tissue sections of both controls were on the same slide as the normally stained sample.

The incubation with the primary antibody was prepared overnight in PBS containing 0.2% (*w/v*) IgG-free BSA at 4 °C. Subsequently, the tissue sections were washed and incubated for 1 h at room temperature (RT) with a secondary antibody (envision kit+ system-HRP labeled polymer anti-mouse; Dako, Jena, Germany). After washing, the sections were revealed using a peroxidase chromogen for immunohistochemistry (IHC) SIGMAFAST 3,3'-Diaminobenzidine (DAB)-tab (Sigma-Aldrich, St. Louis, MO, USA). The nuclei were stained with hematoxylin for 10 s.

2.6. Fluorescent Staining

For the visualization of polySia, instead of the HRP-conjugated secondary antibody, the samples were incubated for 1 h at RT with Alexa Fluor 488-conjugated $(Fab)_2$ fragment of goat anti mouse IgG (H + L) (A11017 Carlsbad, California, CA, USA) (10 µg/mL in 1% BSA) or with Alexa Fluor 568-conjugated goat anti-mouse IgG (H + L) secondary antibody (A11031 Abcam, Cambridge, UK) (1 µg/mL in 1% BSA). For a parallel staining of DDX4, also known as VASA protein, the tissue sections were blocked with PBS containing 5% BSA and goat serum for 1 h at 37 °C. Thereafter, a rabbit IgG polyclonal antibody against DDX4 (ab 13840 Abcam, Cambridge, UK) (1 µg/mL in 1% BSA) was used (overnight at 4 °C). The antibodies bound against DDX4 were labeled with an Alexa Fluor 647-conjugated goat anti-rabbit IgG (H + L) secondary antibody (A212245 Life Technologies, Carlsbad, California) (1 µg/mL in 1% BSA) for 1 h at RT. The DNA was stained with 4',6-diamidino-2-phenylindole (DAPI) for 10 min at RT. Finally, a fixation step was performed using 2% paraformaldehyde (PFA) for 20 min at RT. The stained tissue sections were examined with a laser scanning fluorescence microscope (Carl Zeiss Axio Observer.A1, Oberkochen, Germany).

2.7. Synteny Analysis

For the synteny (blocks of orthologous genes) and paralogy analyses, the *st8sia* gene loci and their neighbor genes were localized using NCBI BLAST search, and the detection of paralogous genes was assessed by manual chromosome walking. We chose genes physically close to the gene of interest to identify only the syntenic segment of interest.

2.8. Statistical Analysis

The calculated values were analyzed with Graph Pad Prism 8.4.3 software (GraphPad Software, San Diego, CA, USA) using a Mann–Whitney test. The differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Polysialylation in Ovary of C. maraena

Kitajima and colleagues found that α2,8-linked polyNeu5Ac was present on salmonid fish eggs [9] and that all polySiaTs were expressed in rainbow trout ovaries [15]. Based on their studies, we investigated the ovaries of *C. maraena*, which also belongs to the salmonid lineage, for α2,8-linked Neu5Ac polymers with a DP ≥ 8 using mAb 735 [21,26]. To this end, ovarian tissue was lysed, and the protein was enriched. After protein separation by SDS-PAGE and Western blotting against polySia, broad signals between 135 and 180 kDa were visualized, which were absent or reduced after endoN treatment (Figure 1). EndoN degrades α2,8-linked Neu5Ac polymers [22,27]. The extensive smear is typical for polysialylated proteins and might result from a heterogeneous polySia chain length distribution on polySia carriers [28,29]. In the case of animal 3, the signal was not completely abolished. This could result from an incomplete digest or a background signal. The visualized protein bands, which showed comparable signal intensities in the endoN-treated and untreated aliquots (low kDa area and in animal 3 proteins > 245 kDa) seem to be the result of an unspecific binding of the applied antibodies. In sum, the obtained results strongly suggest that polySia is present in the ovaries of *C. maraena*.

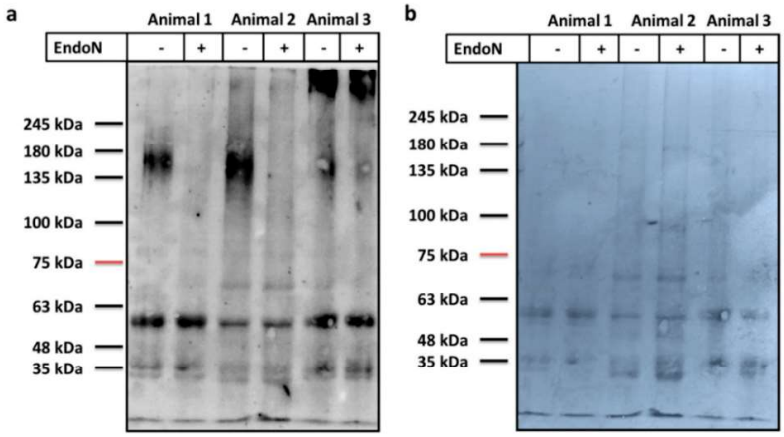

Figure 1. Polysialylation status in maraena whitefish ovary. Proteins (5 µg per lane) were separated by 7% SDS-PAGE, and (**a**) polySia was visualized by Western blotting. An aliquot of each sample was treated with endoN to degrade polySia. The polysialylation status of three independent ovaries (three different animals) is displayed, and molecular masses (kDa) of standard proteins are indicated. (**b**) For loading control, the membrane was stained with Coomassie blue.

It should, however, be noted that it cannot be unambiguously denied that the mAb 735 might additionally visualize other polySia species than α2,8-linked Neu5Ac homopolymers. It has been suggested that anti-polySiaNeu5Ac antibodies (e.g., mAb 12E3) may also bind heteropolymers of

Neu5Ac and Neu5Gc residues, even if the binding efficiency is lower [30,31]. Furthermore, endoN can also degrade Neu5G-containing polySia in addition to Neu5Ac homopolymers but with lower efficiency [30,31].

3.2. Expression Levels of PolySiaT Genes in C. maraena Ovaries

Following the polySia-positive Western blot results, the expression levels of the polySiaT genes were examined using qPCR. In rainbow trout ovaries, three distinct genes are expressed: two *st8sia2* variants and one *st8sia4* gene [15]. A recent phylogenetic analysis suggests that the two *st8sia2* genes resulted from a fourth duplication of the whole genome in Salmonidae (SGD = R4) after the teleost radiation (TGD = R3) [16] (Figure 2a and Supplemental Figure S1). Thus, the genome of maraena whitefish encodes three polySiaTs, just as in rainbow trout.

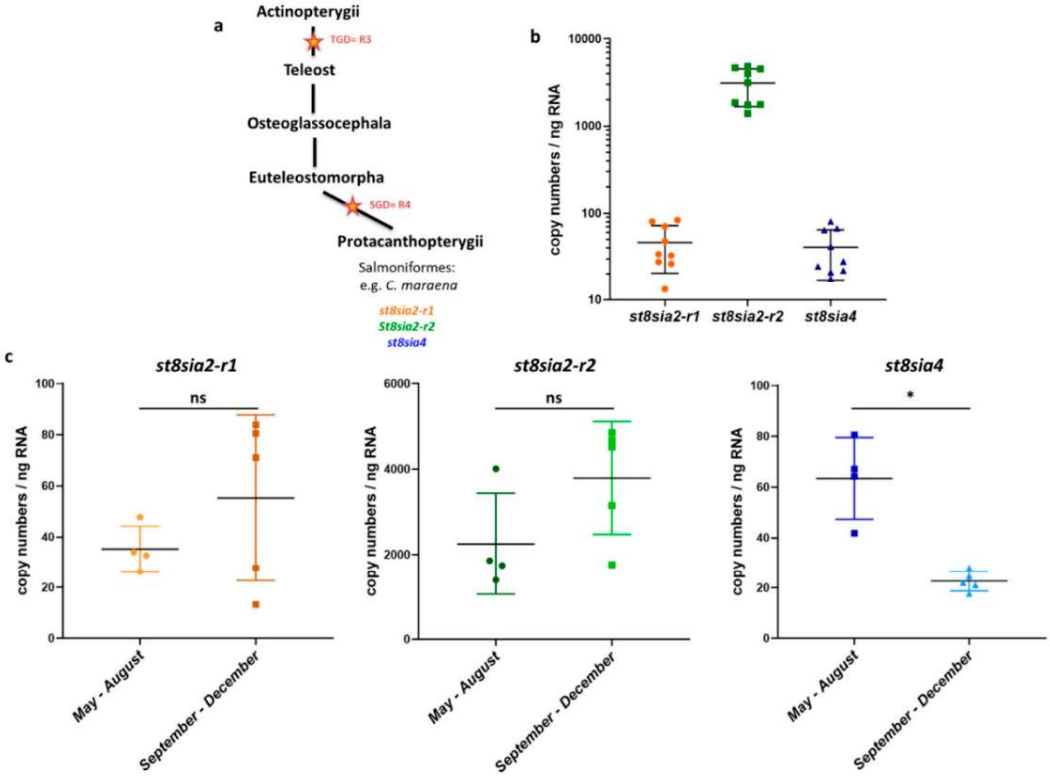

Figure 2. Expression profiling of polysialyltransferase genes in *C. maraena* ovaries. (**a**) Overview of the polyST family evolution in the Protacanthopterygii. Orange stars correspond to the whole-genome duplications (WGDs): R3 (teleost-specific duplication, TGD) and R4 (salmonid-specific duplication, SGD). For a more comprehensive illustration, please see Supplemental Figure S1. (**b**) The transcript levels of *st8sia2-r1* (orange), *st8sia2-r2* (green), and *st8sia4* (blue) were determined in ovaries from maraena whitefish (n = 9 animals). Data are plotted on a logarithmic scale. (**c**) The transcript levels of the polysialyltransferase genes during spring and summer (n = 4 animals) and autumn (n = 5 animals) are separately displayed. All error bars represent the standard deviation. Non-significant, ns; * $p < 0.05$.

To determine the relative transcript levels of the polysialyltransferase genes in the ovaries of *C. maraena*, specific primer pairs for *st8sia2-r1*, *st8sia2-r2*, and *st8sia4* were applied. The qPCR analysis revealed that the numbers of *st8sia2-r1* transcripts (about 46 copies/ng RNA) and *st8sia4* transcripts (about 40 copies/ng RNA) were comparably low (Figure 2b). Remarkably, the number of *st8sia2-r2* transcripts was more than 60 times higher (about 3110 copies/ng RNA).

However, it must be mentioned that the ovaries of the nine whitefish were collected from May until December. In most teleost species, reproduction is cyclic, and seasonality attends several physiological alterations [32]. Concerning the polySiaTs, season-dependent expression is possible in rainbow trout

ovaries, since in September, the mRNA level of *stx-ov* (in *C. maraena st8sia2-r1*) increase and the transcripts of *st8sia4* tend to decrease [15]. For this reason, we separately summarized the determined mRNA levels of the *C. maraena* samples, which were collected during "summer" (from May until August) and "autumn" (from September until December). The mRNA levels for both ST8SiaII enzymes of *C. maraena* slightly increased, although the changes were not statistically significant (Figure 2c). In contrast, the *st8sia4* transcripts slightly decreased.

In sum, the results demonstrate that ST8SiaII-r2 is the dominantly expressed polysialyltransferase in *C. maraena* ovaries.

3.3. Localization of Polysia in Ovaries of Maraena Whitefish

Oogenesis in fish is a dynamic process [33]. Remarkably, oogonia can proliferate throughout the lifetime of fish, and mitosis can take place in mature ovaries, whereas in mammals, its proliferation is restricted to the embryonic and fetal periods [34,35]. Maraena whitefish have asynchronous gonad development, with different cell types being frequently present in close proximity. Figure 3 shows an example of this non-homogeneous ovarial stage organization.

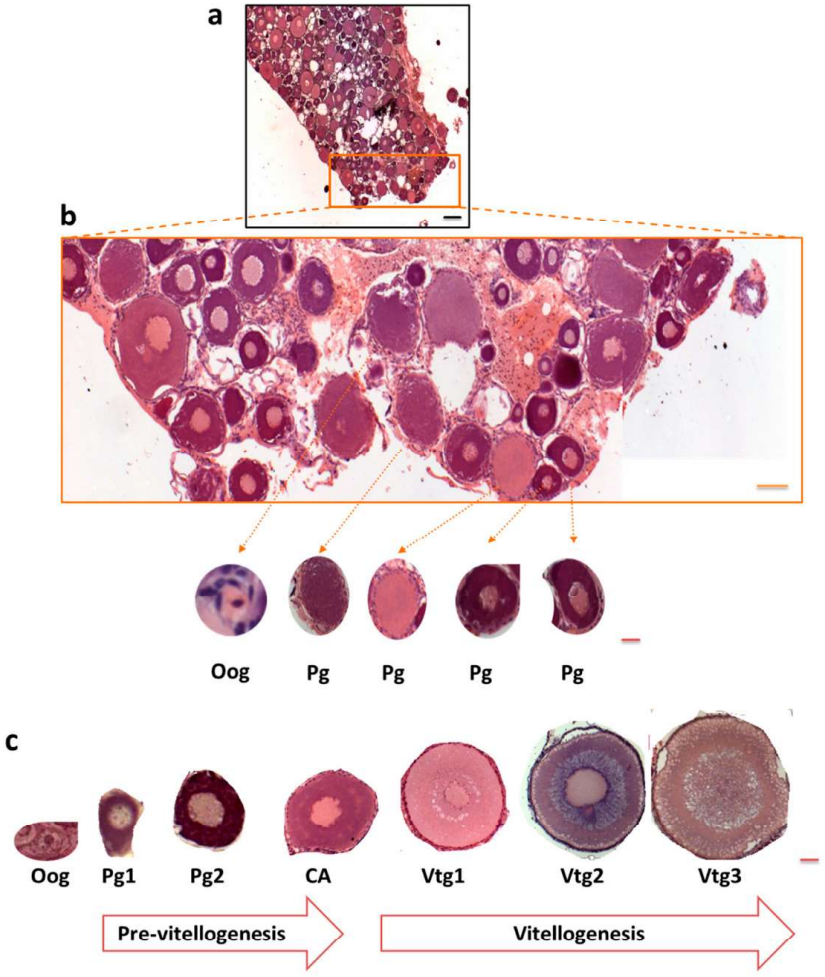

Figure 3. Histological section of *C. maraena* ovary during previtellogenesis. The ovarian tissue was stained with HE; (**a**) black scale bar 200 µm, (**b**) orange scale bar 100 µm, (**b**,**c**) red scale bar 10 µm. The colored box approximately indicates the enlarged areas. (**b**,**c**) Previtellogenesis and vitellogenesis stages in *C. maraena* ovary. The following cells are displayed: oogonia (Oog); oocytes in primordial growth (Pg) and early and late primary growth (Pg1, Pg2, respectively); cortical alveoli (CA); primary, secondary, and tertiary vitellogenesis (Vtg1, Vtg2, and Vtg3, respectively). This schematic presentation of oogenesis is based on [36,37].

To investigate the localization of polySia in ovaries, tissue sections were treated with mAb 735 followed by differently conjugated secondary antibodies (HRP, Figure 4a; Alexa Fluor 488, Figure 4b). The obtained results revealed that polySia-positive cell clusters are mainly localized between cell units in previtellogenic stages (Figure 4). The staining did not take place when polySia was degraded with endoN.

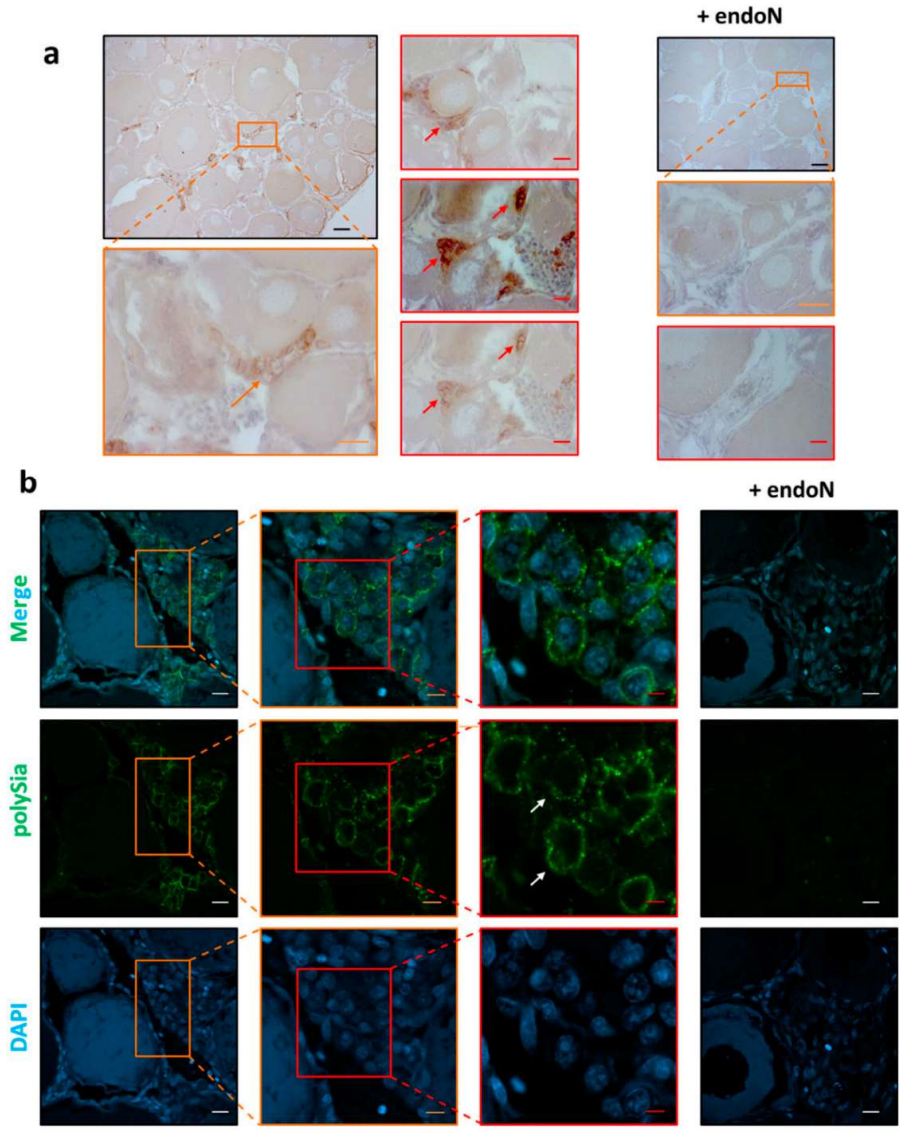

Figure 4. Histological analysis of the polysialylation status in ovaries from *C. maraena*. mAb 735 was applied to detect polySia. (**a**) For immunohistochemical visualization, an HRP-conjugated secondary antibody was used. For negative control, polySia was degraded by endoN. Nuclei were stained with hematoxylin. Selected polySia-positive cells are indicated with orange and red arrows. Black scale bars: 100 µm; orange scale bars: 20 µm; red scale bars: 10 µm. For polySia staining and the respective endoN-digest on serial sections, please see Supplemental Figure S2a. (**b**) PolySia staining was performed with a combination of mAb 735 against polySia and an Alexa Fluor 488-conjugated secondary antibody (green). Controls were performed by depolysialylating with endoN (+ endoN). Nuclei were stained with DAPI (blue). The extensive DAPI staining in higher stages of egg development is common [38]. Selected polySia-positive cells are labeled with white arrows showing, in contrast to higher stages of egg development, a typical nuclear staining. White scale bars: 20 µm; orange scale bars: 10 µm; red scale bars 5 µm. The colored boxes approximately indicate the enlarged areas.

Interestingly, Nakamura et al. found that oogonia in *Oryzia latipes* ovaries formed clusters of proliferating cells between cells in previtellogenic stages, which can be stained with antibodies against DEAD-box helicase proteins [35]. Based on these studies, we used polyclonal antibodies against DEAD-box helicase 4 (DDX4). In parallel, polySia was visualized. The results demonstrate that several DDX4-positive cells are polySia positive (Figure 5) and suggest that oogonia nests might be a source of polySia in the analyzed ovaries of maraena whitefish.

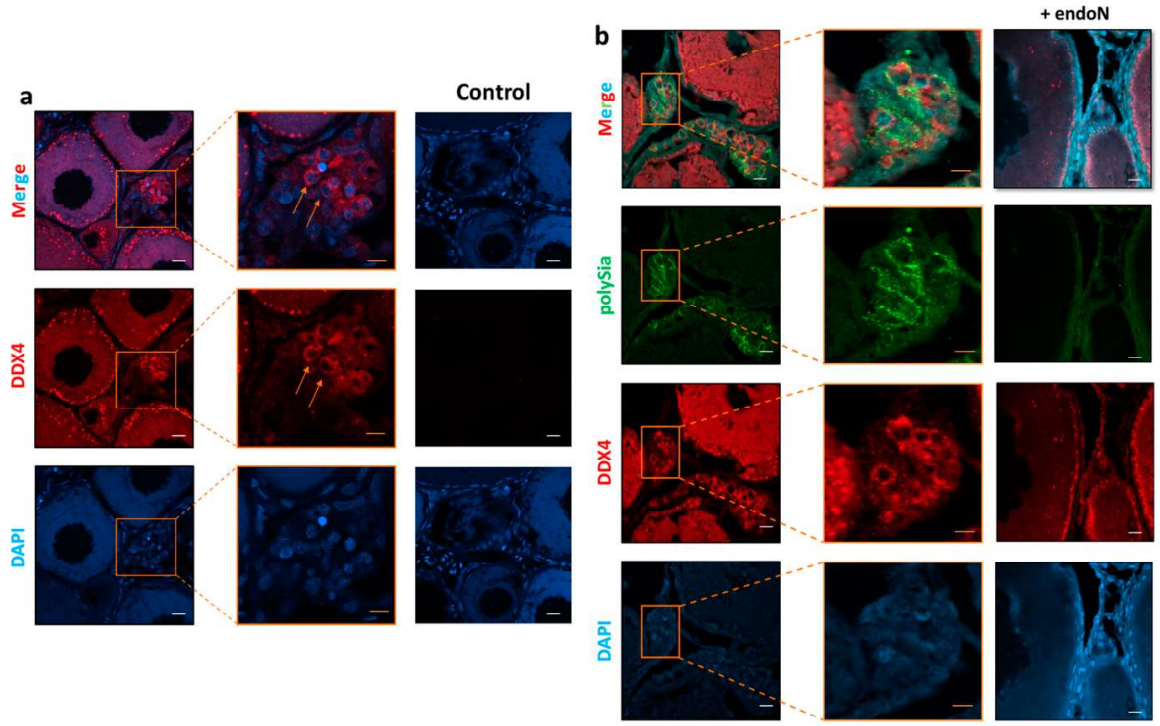

Figure 5. Localization of oogonia and polySia in *C. maraena* ovary. (**a**) DDX4 staining was performed with a DDX4 antibody and an Alexa Fluor 647-conjugated secondary antibody (red). Selected oogonia are indicated with orange arrows. Controls were performed by omitting the first antibody (control). (**b**) PolySia was stained in parallel using a combination of mAb 735 against polySia and an Alexa Fluor 488-conjugated secondary antibody (green). For negative control, polySia was degraded by endoN. Nuclei were stained with DAPI (blue). White scale bars: 20 µm; orange scale bars: 10 µm. The colored boxes approximately indicate the enlarged areas.

The heterogeneous distribution of polySia-positive cell clusters in the tissue sections explains the high level of variance in the polyST expression levels (Figure 2) and that the intensity of polySia Western blot signals (Figure 1) are not identical in all animals. The tissue parts of ovaries will generally have a different composition of polySia-positive and -negative cells.

3.4. Polysialylation in Ovaries of S. lucioperca

In *C. maraena* ovaries, with ST8SiaII-r2, only one of the three polySTs (ST8SiaII-r1, ST8SiaII-r2, and ST8SiaIV) was expressed with copy numbers higher than 1000 (Figure 2b). The other two, ST8SiaII-r1 and ST8SiaIV, seemed present at significantly lower levels (less than 100 copy numbers per ng RNA). Thus ST8SiaII-r2 may synthesize the most polySia chains, which are present in clusters of oogonia in *C. maraena*. However, it is possible that ST8SiaII and IV are cooperatively involved in polysialylation, as hypothesized for rainbow trout ovaries [15]. Interestingly, in other teleost lineages, such as Neoteleostei, ST8SiaIV was lost and only one variant of ST8SiaII is present (Figure 6a and Supplemental Figure S1). Consequently, the availability of various polySiaTs is restricted to ST8siaII.

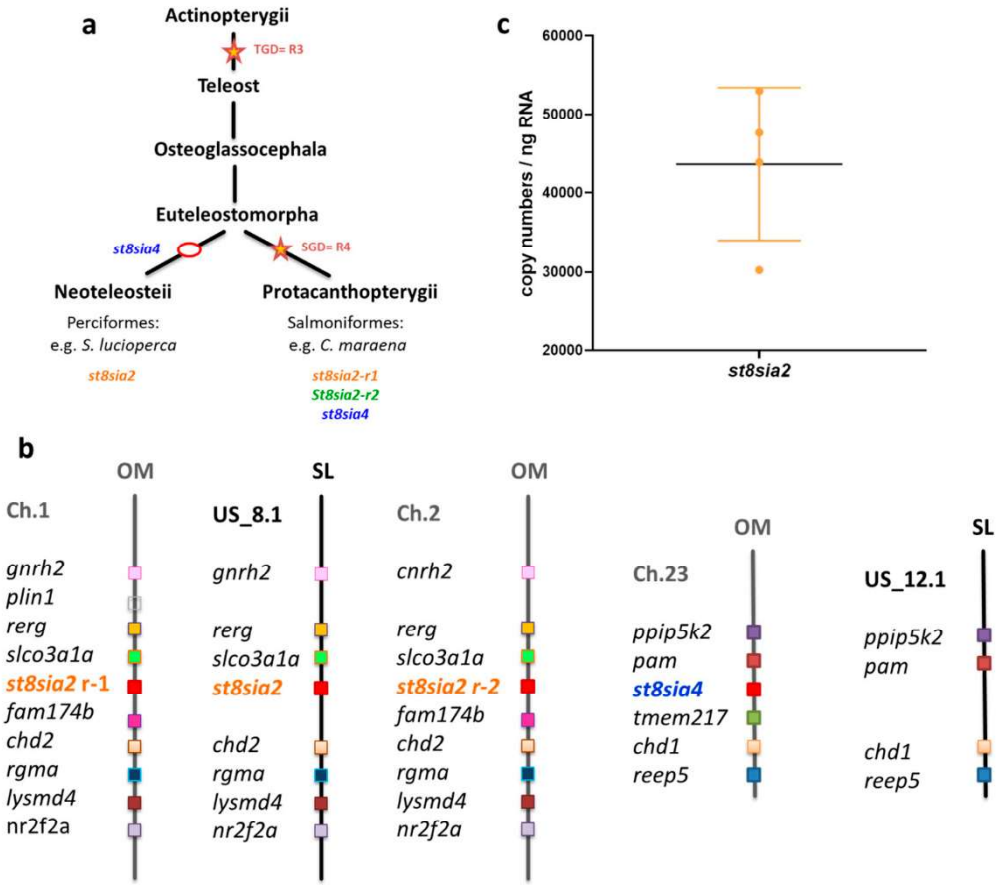

Figure 6. Expression level of *st8sia2* in ovaries from *S. lucioperca*. (**a**) Overview of the polyST family evolution in Actinopterygii. Orange stars correspond to the WGDs, and red circles indicate the loss of *st8sia4*. R3 (teleost-specific duplication, TGD) and R4 (salmonid-specific duplication, SGD). For a more comprehensive illustration, please see Supplemental Figure S1. (**b**) Schematic representation of the chromosomal location of *st8sia* genes, syntenic relationships of polySiaTs, and the neighboring gene loci retrieved from rainbow trout (*O. mykiss*, OM) and pike perch (*S. lucioperca*, SL). The orthologues were determined using information from ENSEMBL and NCBI. (**c**) The transcript level of *st8sia2* was determined in *S. lucioperca* ovaries (n = 4 animals).

We conducted synteny and paralogy analyses of the *st8sia2-r1* and *st8sia2-r2* loci in genomes of Salmonidae. Figure 6c illustrates the presence of a paralogon including these two gene loci and *gnrh*, *rerg*, *slco3a1a*, *fam174b*, *chd2*, *rgma*, *lysmd4*, and *nr2f2a* paralogues on chromosomes 1 and 2 in the rainbow trout *O. mykiss*. This series of genes is well conserved in the pike perch *S. lucioperca* on scaffold (NW_022173278.1), further suggesting that the *C. maraena st8sia2-r1* and *st8sia2-r2* are co-orthologues of the *S. lucioperca st8sia2* and could have the same function in fish ovaries.

In *S. lucioperca* ovary samples, the Q-PCR analysis of the *st8sia2* expression levels revealed that it is more than 10 times higher (about 48,133 copies/ng RNA) than *st8sia2-r2*, the dominant polysialyltransferase in maraena whitefish ovaries (Figure 6c). This might be a compensatory effect of the loss of ST8SiaIV and the absence of a second ST8SiaII polysialyltransferase. However, it must be mentioned that the environmental/breeding conditions of *C. maraena* were different compared to *S. lucioperca*. Thus, a quantitative comparison is hardly possible. Nevertheless, it is obvious that, also in *S. lucioperca* ovaries, polysialyltransferases are expressed.

Subsequently, the polysialylation status of *S. lucioperca* ovaries was also investigated by immunohistochemistry. Consistent with the results from *C. maraena*, in *S. lucioperca*, clustered cell populations were polySia positive (Figure 7). Ovarian tissue sections from these organisms were also stained against polySia and DDX4 in parallel. As illustrated in Figure 8, several oogonia also

displayed a co-staining of polySia and DDX4 in *S. lucioperca*. Thus, despite the absence of ST8SiaIV, polySia is synthesized in these cell populations, likely by the ST8SiaII enzyme.

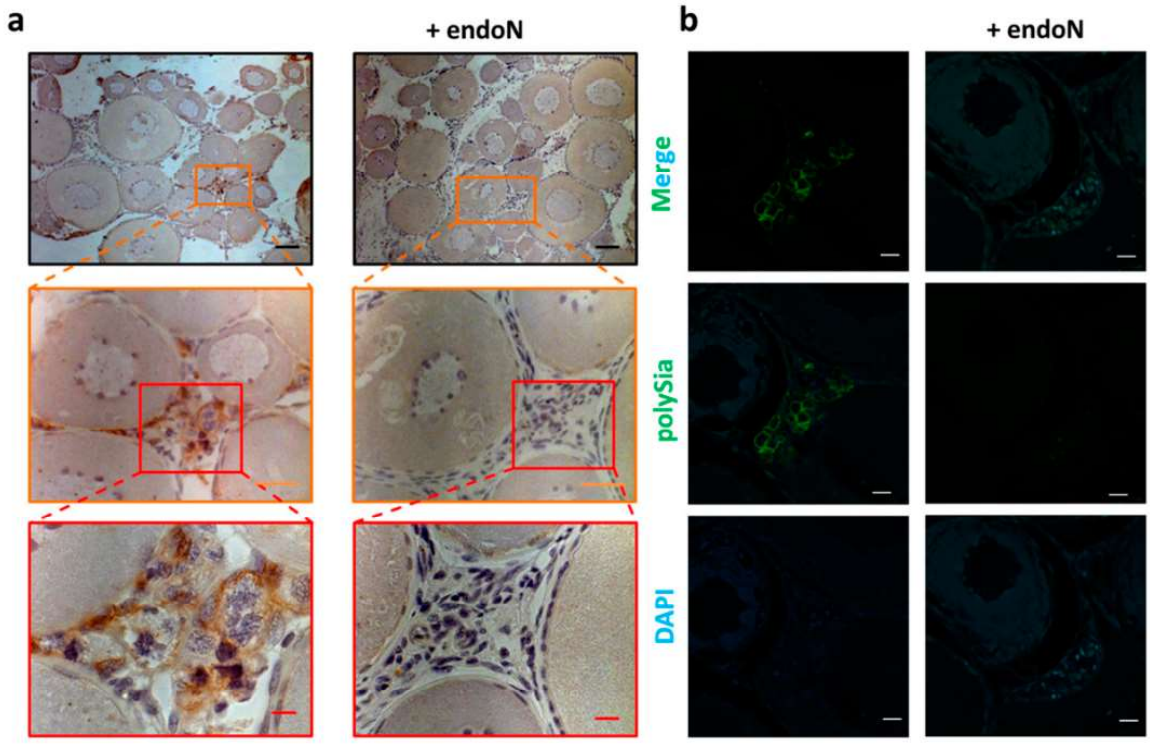

Figure 7. Localization of polySia in ovaries from *S. lucioperca*. mAb 735 was applied to detect polySia in *S. lucioperca* ovary tissue in the previtellogenesis stage. For negative control, polySia was degraded by endoN. (**a**) For immunohistochemical visualization, an HRP-conjugated secondary antibody was used. Nuclei were stained with hematoxylin. Black scale bar: 100 µm; orange scale bar: 20 µm; red scale bar: 10 µm. For polySia staining and the respective endoN-digest on serial sections, please see Supplemental Figure S2b. (**b**) PolySia staining was performed with a combination of mAb 735 and an Alexa Fluor 488-conjugated secondary antibody (green). Nuclei were stained with DAPI (blue). White scale bars: 20 µm. The colored boxes approximately indicate the enlarged areas.

The heterogeneity of polySia-positive and -negative cells in a cell population is common and depends very often on the state of activation [2–4,39]. In the case of neuronal cells, for instance, polySia supports the migration of the cells [40] or influences access to growth factors [41]. Since in the ovaries numerous remodeling processes take place, comparable polySia-influenced mechanisms are conceivable. However, the obtained results do not enable valid conclusions to be drawn.

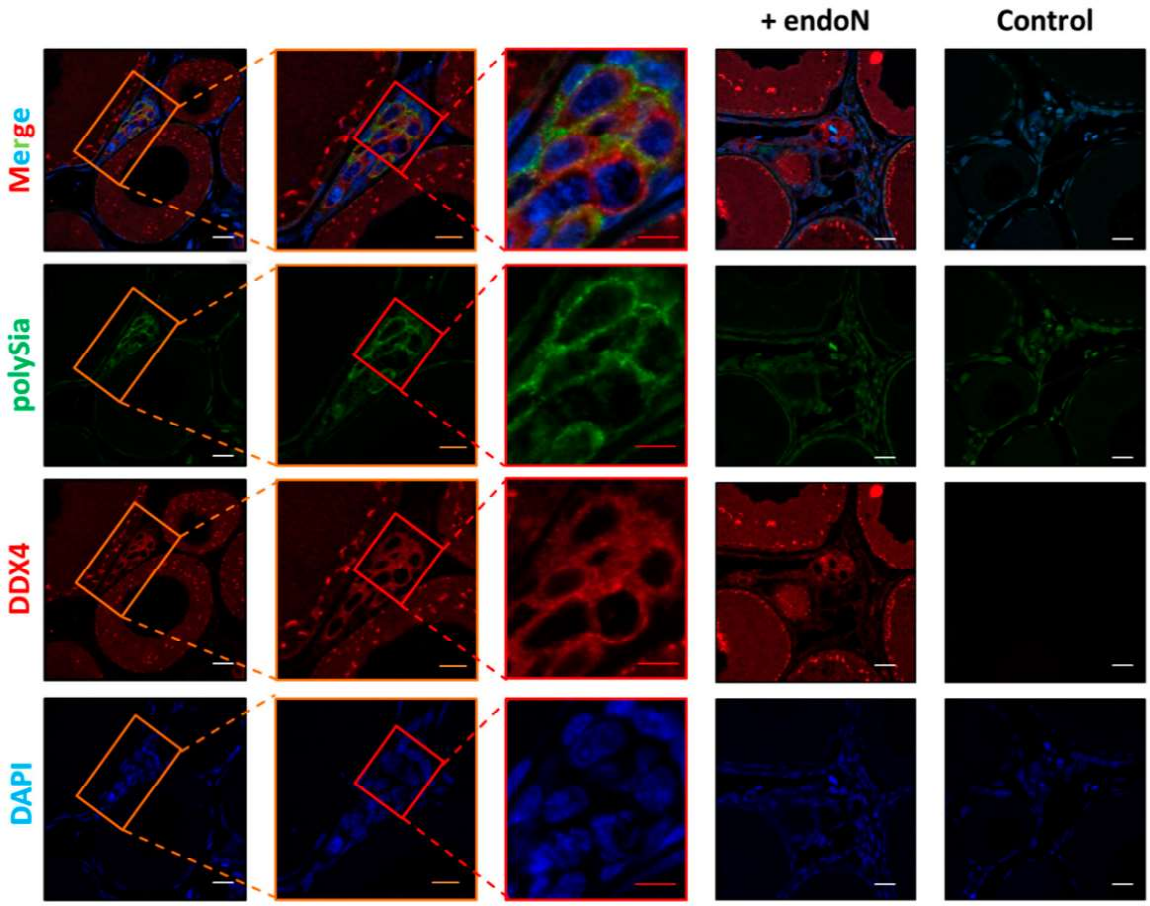

Figure 8. Localization of DDX4 and polySia in *S. lucioperca* ovary. PolySia staining was performed with a combination of mAb 735 against polySia and an Alexa Fluor 568-conjugated secondary antibody (green). For negative control, polySia was degraded by endoN. Germ cells were visualized with a polyclonal antibody against DDX4 in combination with an Alexa Fluor 647-conjugated secondary antibody (red). Controls were performed by omitting the first antibodies (control). Nuclei were stained with DAPI (blue). White scale bars: 20 μm; orange scale bars: 10 μm; red scale bars 6 μm. The colored boxes approximately indicate the enlarged areas.

4. Conclusions

A special characteristic of dynamic oogenesis in fish is the possibility of oogonia proliferating throughout the fish lifetime [31]. Intriguingly, our data revealed that in Salmonidae and Percidae, clusters of these cells exhibited polySia on their cell surfaces, although the fish families had different setups of polySiaTs. Whereas the Salmonidae genome contained three genes for polySiaTs, namely *st8sia2-r1*, *st8sia2-r2*, and *st8sia4*, Percidae could only use *st8sia2*. Interestingly, the *st8sia2* gene in Percidae was flanked by genes comparable to *st8sia2-r1 and st8sia2-r2* in Salmonidae. In line with this observation, in *C. maraena*, the mRNA levels of *st8sia2-r2* were 60 times higher than the values for *st8sia2-r1* and *st8sia4*. Thus, it seems that polysialylation of oogonia is driven by ST8SiaII variants in both fish families and that polySia is involved in the cellular processes of germ cells during oogenesis in fish. Altogether, these observations further suggest a conserved function for polysialylation found in fish oogonia. Since it is well known that polySia is essentially involved in migration and proliferation processes of neural precursor cells [39], a comparable function in oogonia seems to be likely.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4409/9/11/2391/s1, Figure S1: Scenario illustrating the evolutionary history of the polysialyltransferase genes in Salmoniformes and in Perciformes; http://www.mdpi.com/2073-4409/9/11/2391/s2, Figure S2: Histological analysis of the polysialylation status in ovaries from *C. maraena* and *S. lucioperca*.

Author Contributions: M.T.V. performed the Western blot and immunohistochemistry experiments; J.M.-R. conducted the RT-qPCR analyses; J.M.-R. and M.T.V. performed the statistical analyses; A.R., A.H.-L., and S.P.G. supervised the experiments; R.B. provided the Maraena Whitefish samples; M.T.V. and S.P.G. wrote the paper; all authors edited the manuscript. All authors have read and agreed to the final version of the manuscript.

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5.3 Study III: Time-Dependent Effects of Acute Handling on the Brain Monoamine System of the Salmonid Coregonus Maraena

Martorell-Ribera J., **Venuto M.T.**, Otten W., Brunner R.M., Goldammer T., Rebl A. Gimsa U. *Front*. **2020**; 14. (https://doi.org/10.3389/fnins.2020.591738)

Brief summary:

In this study, a pseudo wild Salmonidae fish from the Baltic Sea, *C. maraena*, was chosen as a novel aquaculture species model to investigate the influence of stress on the immune response. Considering the brain as the control centre of the stress response, the aim of this study is to analyses of the effects of acute and repeated handling on the brain monoaminergic systems of *C. maraena.*

Three stress handlings have been performed (1 minute per day), and the analyses were made after 3 h, 24 h and 10 days post-repeated stress. A group of genes were used as potential biomarkers to measure the handling stress. Plasma cortisol was quantified in order to figure out the activation of the stress axis. Moreover, genes related to the monoamines system were analysed due to the markers of stress.

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Time-Dependent Effects of Acute Handling on the Brain Monoamine System of the Salmonid *Coregonus maraena*

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The immediate stress response involves the activation of the monoaminergic neurotransmitter systems including serotonin, dopamine and noradrenaline in particular areas of the fish brain. We chose maraena whitefish as a stress-sensitive salmonid species to investigate the influence of acute and chronic handling on the neurochemistry of monoamines in the brain. Plasma cortisol was quantified to assess the activation of the stress axis. In addition, we analyzed the expression of 37 genes related to the monoamine system to identify genes that could be used as markers of neurophysiological stress effects. Brain neurochemistry responded to a single handling (1 min netting and chasing) with increased serotonergic activity 3 h post-challenge. This was accompanied by a modulated expression of monoaminergic receptor genes in the hindbrain and a significant increase of plasma cortisol. The initial response was compensated by an increased monoamine synthesis at 24 h post-challenge, combined with the modulated expression of serotonin-receptor genes and plasma cortisol concentrations returning to control levels. After 10 days of repeated handling (1 min per day), we detected a slightly increased noradrenaline synthesis and a down-regulated expression of dopamine-receptor genes without effect on plasma cortisol levels. In conclusion, the changes in serotonergic neurochemistry and selected gene-expression profiles, together with the initial plasma cortisol variation, indicate an acute response and a subsequent recovery phase with signs of habituation after 10 days of daily exposure to handling. Based on the basal expression patterns of particular genes and their significant regulation upon handling conditions, we suggest a group of genes as potential biomarkers that indicate handling stress on the brain monoamine systems.

Keywords: catecholamines, marker genes, monoamine receptors, salmonids, serotonin, stress

Abbreviations: 5-HIAA, 5-hydroxyindoleacetic acid; 5-HT, 5-hydroxytryptamine; BSC, brain-sympathetic-chromaffin axis; DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; FB, forebrain; MB, midbrain; HB, hindbrain; HPA, hypothalamic-pituitary-adrenal axis; HPI, hypothalamic-pituitary-interrenal axis; HPLC, high-pressure liquid chromatography; HVA, homovanillic acid; LC, *locus coeruleus*; NA, noradrenaline; NLV, *nucleus lateralis valvulae*; POA, pre-optic area; STR, *striatum*.

INTRODUCTION

The immediate stress response involves the activation of the brain monoaminergic systems, including serotonin (5-hydroxytryptamine; 5-HT), DA and NA as major neurotransmitters. Depending on the type of stressor and the duration and intensity of its occurrence (Barton, 2002), these monoamines modulate the neuronal responses in particular areas of the brain, principally the telencephalon, hypothalamus and brain stem (Winberg and Nilsson, 1993; Kaslin and Panula, 2001). In essence, monoamines affect behavior, the formation of memory and the activity of the brain regions that initiate the neuroendocrine stress axes (Feldman et al., 1995; Viltart and Vanbesien-Mailliot, 2007; Lõrincz and Adamantidis, 2017) to reprogram metabolism, immunity, growth and reproduction (Silbergeld, 1974; Hemre and Krogdahl, 1996). This complex response and the associated physiological changes aim to cope with environmental and/or anthropogenic challenges to reinstall homeostasis (Schreck et al., 2016). Handling procedures are a common anthropogenic disturbance in aquaculture. They activate the stress response in various fish species reflected by increased plasma cortisol concentrations (Barton et al., 1987; Barcellos et al., 2011), altered gene expression in the brain in a specific time-course (Krasnov et al., 2005) and elevated monoaminergic activity in particular brain regions (Gesto et al., 2013). The initial stress response of fish is translated into a systemic stress response via the hypothalamus. It directs the endocrine reaction by initiating the "fight-or-flight response" through the BSC axis (Feldman et al., 1995). This triggers the release of the catecholamines adrenaline and noradrenaline from the head kidney (Reid et al., 1998). Subsequently, the initiation of the BSC axis is followed by the activation of the HPI axis, which causes the release of cortisol into the circulation (Wendelaar Bonga, 1997; Schreck et al., 2016). Eventually, the HPI and the BSC induce a series of compensatory physiological processes to direct the body's metabolic resources to survival needs (Irwin and Cole, 2013; Aerts, 2018).

Monoaminergic neurons project into wide areas of the brain and in particular to those regions that form the limbic system, which evaluates sensory inputs such as visual or olfactory cues as possible threats (Vuilleumier, 2005) and initiates the central stress response with monoamines as active messengers (Morgane et al., 2005). Important monoaminergic neuron clusters are situated in the fish hypothalamus, a control center of the limbic system (Kaslin and Panula, 2001). In mammals, the limbic system is composed of the hypothalamus, hippocampus, amygdala and thalamus, while it is spread between the telencephalon and the midbrain of teleost fishes (Winberg and Nilsson, 1993; Panula et al., 2010; Mueller, 2012) (**Figure 1**).

Upon synaptic release, monoamines bind to their respective receptors on the target cell. Depending on the type of receptor, this binding can activate or inhibit neuronal functions (Flügge, 1999; Nichols and Nichols, 2008; Mishra et al., 2018). In mammals, several types of receptors for 5-HT (5-HT-1-7), dopamine (D1-5), adrenaline and NA (α1, α2 and β) have been identified (Flügge, 1999; Nichols and Nichols, 2008; Maximino and Herculano, 2010; Fröhlich, 2016; Mishra et al., 2018). However, there is little information on their orthologs in fish. For instance, the adrenergic receptor α2d is not present in mammals, but it is strongly expressed in salmonids (Martorell-Ribera et al., 2020).

In the fish brain, monoamines play an important role in the mechanisms of stress coping. In particular, 5-HT induces different activity patterns in proactive and reactive salmonids (Winberg et al., 1992; Øverli et al., 1999, 2001). In addition, the genes encoding 5-HT receptors (*HTR1A*α and β) in the telencephalon of rainbow trout *Oncorhynchus mykiss* are down-regulated by stress (Moltesen et al., 2016). Such a downregulation of 5-HT receptors may reduce 5-HT uptake and lead to increased 5-HT-metabolism (Dwarkasing et al., 2016). In line with this, the chasing of rainbow trout increased the concentrations of 5-HIAA (the main 5-HT metabolite) in the telencephalon and hypothalamus (Gesto et al., 2013). DA has also an important role in modulating behavioral responses. For example, increased dopaminergic activity in the telencephalon of fish has been linked to avoidance behavior (Höglund et al., 2005) and reward (Teles et al., 2013). The expression of the dopamine receptor 2 (DRD2) gene was up-regulated in the brain of bold zebrafish *Danio rerio* compared to reactive individuals (Thörnqvist et al., 2019). Furthermore, handling and treatment with anesthetics increased the concentrations of DA metabolites in the telencephalon of Arctic charr *Salvelinus alpinus* (Backström et al., 2017) comparable with the aforementioned 5-HT levels in stressed rainbow trout. Also NA, which contributes to vigilance and arousal (Singh et al., 2015), has been subject to increased turnover in rainbow trout under stress conditions (Øverli et al., 2001). In Arctic charr, agonistic interactions increased NA concentrations in the telencephalon of dominant individuals (Backström et al., 2015). Taken together, the observed increase in monoamine metabolites is an indicator for the stress response in different salmonid species.

Maraena whitefish *Coregonus maraena* (Bloch) is a salmonid fish present in the Baltic region in anadromous and landlocked populations (Kottelat and Freyhof, 2007). In Germany, it has been reared for intensive aquaculture production since 2005 (Jansen et al., 2008). Our previous studies revealed that maraena whitefish is highly sensitive to stressors compared to salmonids that are better adapted to husbandry conditions, such as rainbow trout or Atlantic salmon *Salmo salar* (Altmann et al., 2016; Korytář et al., 2016; Rebl et al., 2018). This study investigates the influence of acute and repeated (chronic) handling stress on the monoaminergic systems in different brain regions of maraena whitefish. We focused our research on stress-related changes in plasma cortisol concentrations, monoamine neurotransmitters and metabolites and the expression profile of monoamine-related genes in the brain. Our specific aims were to elucidate (i) which brain regions were activated during handling; (ii) which monoaminergic systems showed increased sensitivity to this type of stressor; (iii) how handling can affect HPI activation; (iv) whether a single episode of handling was quickly overcome or whether it can have long-lasting effects on the monoamine neurochemistry of maraena whitefish and (v) how

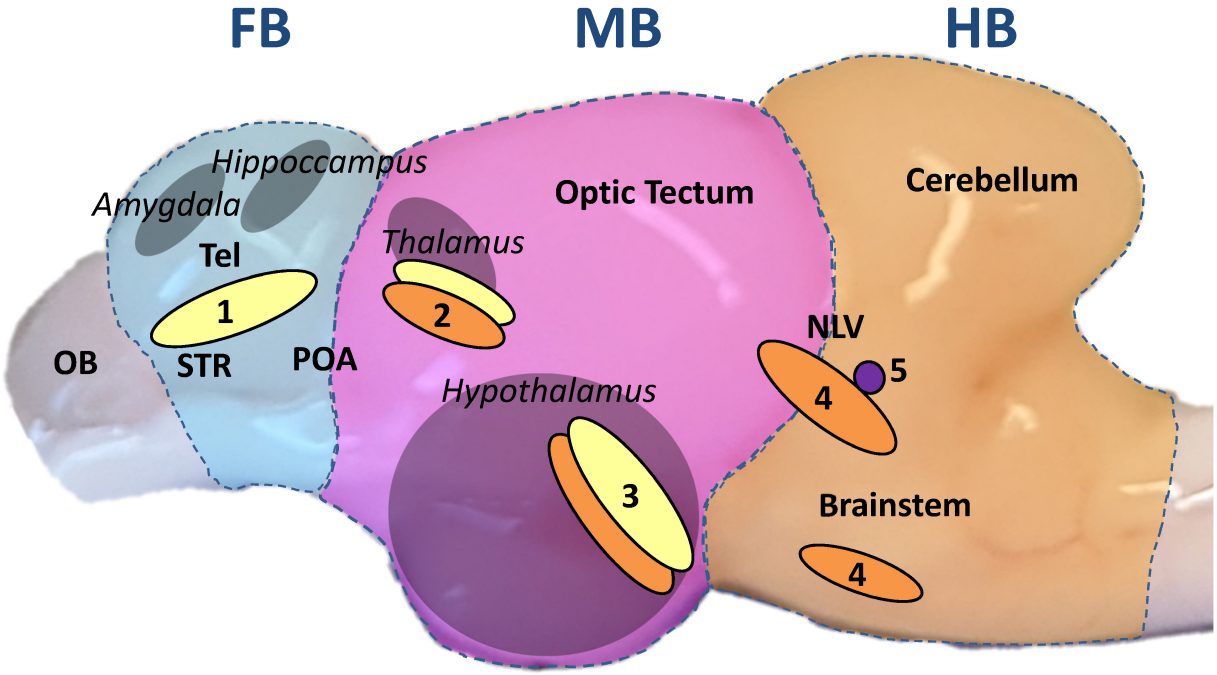


FIGURE 1 | Sagittal view of the whitefish brain. The brain was divided transversally, perpendicular to the rostral-caudal axis, in three parts throughout the experiment: forebrain section (FB, blue underlay); midbrain section (MB, pink); hindbrain section (HB, orange). The FB comprised the telencephalon (dorsal and ventral). The homologous regions to the mammalian amygdala and hippocampus are situated in the dorsal telencephalon while the ventral telencephalon comprises the equivalent to the *striatum* (STR) and dorsally the preoptic area (POA) as described by Mueller (2012); Vindas et al. (2017); Geng and Peterson (2019). The olfactory bulb (OB) was discarded. The MB included every region between optic tectum and diencephalon with the inferior lobes of the hypothalamus from a dorsal-ventral view. The pretectal complex, where the teleost thalamus has been described, is located in the dorsal part of the section (Kaslin and Panula, 2001; Mueller, 2012). The HB was composed of the cerebellum and the brainstem, including the *nucleus lateralis valvulae* (NLV), which was most probably partially divided between MB and HB during dissection (Gaspar and Lillesaar, 2012). From a transversal view, the anterior edge of the cerebellum marked the sectioning line on the brain stem. The different parts of the limbic system are marked in gray and the names in italics. Based on the aforementioned publications on teleost brain organization, monoaminergic neuron populations are marked in yellow for DA neurons; orange for 5-HT neurons and purple for NA neurons: (1) telencephalic complex; (2) pretectal complex; (3) hypothalamic paraventricular nucleus; (4) anterior *raphe nuclei*; (5) *locus coeruleus*.

the monoaminergic system responded over time to repeated episodes of handling. Furthermore, we sought to identify genes that might be suited as animal-based biomarkers for indicating stress in different regions of the brain.

MATERIALS AND METHODS

Husbandry of Maraena Whitefish

The Institute for Fisheries of the State Research Center for Agriculture and Fishery Mecklenburg-Western Pomerania (Born, Germany) and BiMES - Binnenfischerei GmbH (Friedrichsruhe, Germany) provided maraena whitefish for this study. Fish were reared in fresh-water recirculation systems with a stocking density of 10 kg/m^3 at 18°C and a 12:12-h day-night cycle. Water quality was maintained by automated purification and disinfection (bio-filter and UV light). The concentrations of NH_4^+, NO_2^-, NO_3^-, and NH_3 in the water, pH, temperature and oxygen saturation were constantly recorded. Commercial dry pellets (4.5 mm; Biomar, Inicio Plus, Aarhus, Denmark) were distributed by automatic feeders at a daily rate of 0.8–4.0% of the biomass of maraena whitefish in the tank.

Acute and Chronic Handling Experiments

All procedures have been approved by the Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei, Mecklenburg-Vorpommern, Germany (LALLF M-V/TSD/7221.3-1-069/18). The fish (n = 48 in total) used in the experiments were juvenile with a starting size of about 20 cm. They were allowed to acclimatize to the recirculation system for at least 2 weeks in the reservoir tank (500 l) and were transferred to the experimental tanks 7 days before the experiments started. These tanks were identical dark polyethylene cylinders with a capacity of 150 L. Experiments were performed in the morning (between 8 and 11 a.m.) to minimize the influence of circadian rhythms on the measurements.

Each of the acute handling experiments started with eight rearing tanks with one pair of fish per tank, and four identical secondary tanks without fish (**Figure 2**). The fish were placed in the tanks in pairs to ensure that the sampling and treatment procedures disturbed the other animals that were part of the experiment as little as possible. At the start of the experiment and before the handling procedure, one fish per pair was euthanized. This fish was designated the control fish (0 h). After sampling of the control fish, the handling protocol was applied for 1 min

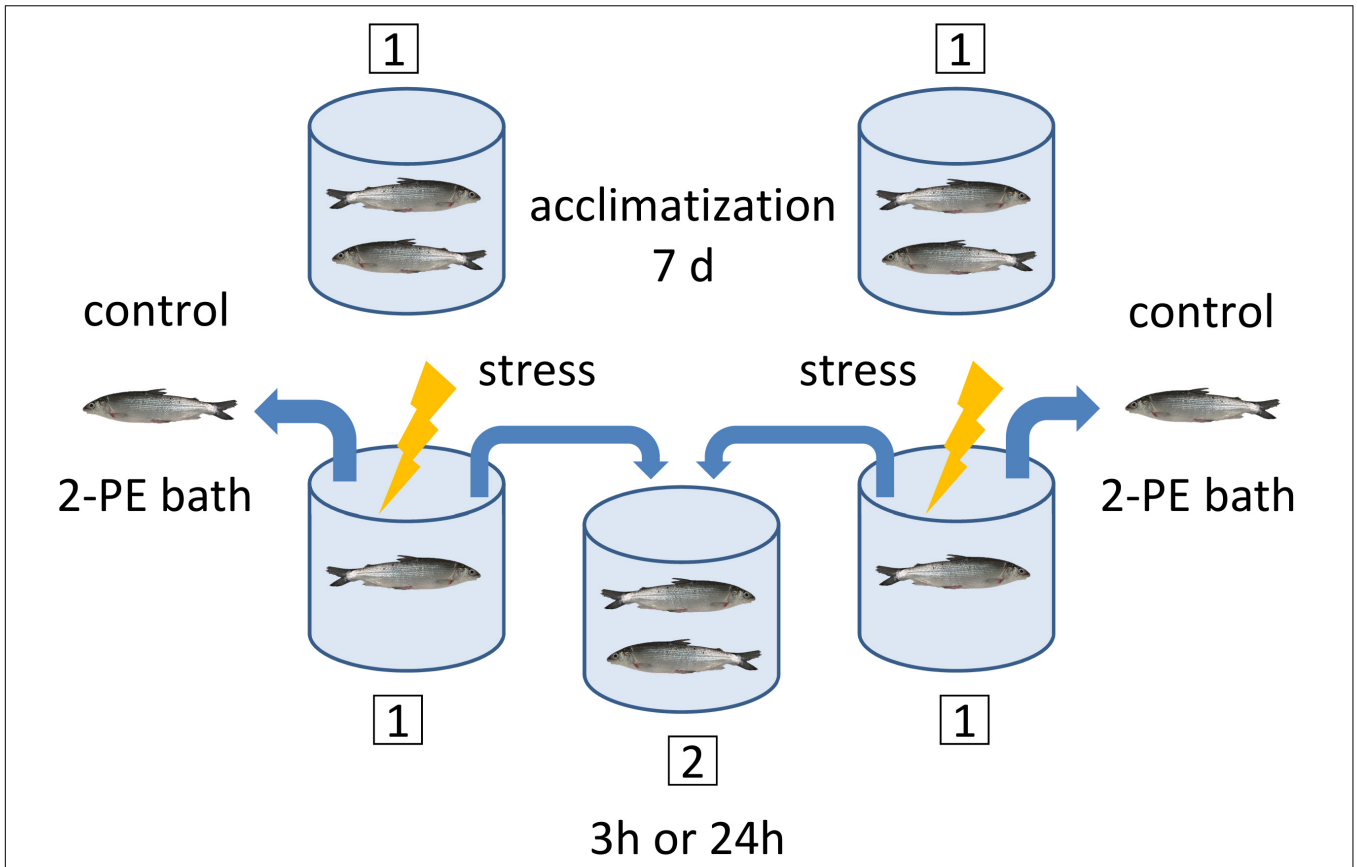


FIGURE 2 | Sampling and handling protocol for the acute experiments. A pair of maraena whitefish was placed in each experimental tank (1) and left undisturbed for acclimatization during 7 days prior to sampling. At day 7, the control fish were sampled for the 3 h or 24 h experiments before stress was applied to the test fish. The timing was as follows: The control fish were caught and placed in a 2-phenoxyethanol (2-PE) bath for anesthetic overdosage, which takes approximately 2–5 min. While the control fish were under anesthesia, the test fish remaining in the tank were subjected to the handling procedure treatment. The test fish were then transferred to the secondary tank (2), where they remained either 3 h or 24 h until sampling. This procedure was applied to two primary tanks simultaneously, where at the end two control fish were in the 2-PE bath and two test fish were transferred to the same secondary tank. The maraena whitefish were always in pairs of two, except for the handling procedure (1 min), during which they were alone in the tank.

to the remaining fish, which was designated as test fish. The handling protocol consisted of hunting and catching fish with nets, intermittently lifting the fish out of the water and finally transferring them to a secondary tank, where test fish pairs were left undisturbed for either 3 h or 24 h. After this period, test fish were euthanized and tissues samples were taken as described below (see section "Sampling of Tissues").

For the chronic handling experiment, control fish (n = 8) and test fish (n = 8) were kept in pairs in the same condition in eight separate tanks for the duration of the experiment. The experiment was divided into two rounds of eight fish each to swap the tanks for the test fish with the tanks for the controls. The test fish were hunted and netted once a day for 1 min for 10 days, while the controls were left undisturbed. The net lifting and tank-transferring steps performed in the acute-handling procedure were skipped in the chronic handling experiment to avoid skin injuries. To minimize habituation, handling was always performed at a random time within the 12 h 'daylight' period. The fish were euthanized 10 days after the onset of the chronic handling procedures, 24 h after the last handling procedure.

Sampling of Tissues

Before tissue sampling, fish were euthanized with an overdose of 2-phenoxyethanol (0.7 ml/l) followed by spine dissection at the skull level. Sampling and killing methods followed the standards described in the German Animal Welfare Act [§4(3) TierSchG]. Immediately after killing, blood was drawn from the caudal vein using a heparinized syringe and centrifuged at 270 × g for 15 min at 6°C to obtain plasma, which was stored at −80°C. Subsequently, the fish brain was quickly and carefully dissected in three parts transversally and perpendicular to the rostral-caudal axis: forebrain section (FB), midbrain section (MB), and hindbrain section (HB). **Figure 1** describes in detail the cutting lines of the dissection and the brain parts used in this study. The FB included the telencephalon (dorsal and ventral), while the olfactory bulb was discarded. From the dorsal-ventral view, the MB included each region between the optic tectum and the inferior lobes of the hypothalamus. The HB was composed of the cerebellum and the brainstem. In a transverse view, the anterior edge of the cerebellum marked the line of incision at the brainstem, thereby excluding the spinal cord. The three

different sections were separately weighed and homogenized in 500 µl ice-cold PBS. The resulting homogenate was divided into two aliquots and snap-frozen in liquid nitrogen for RNA isolation or monoamine analysis (see sections "Quantification of Monoamines and Metabolites" and "RNA Isolation and cDNA Synthesis").

Measurement of Plasma Cortisol

The cortisol concentration in plasma was quantified by a competitive enzyme-linked immunosorbent assay (ELISA; Cusabio Technology, Houston, TX, United States) according to the manufacturer's instructions. The level of absorbance was measured at 450 nm in a Beckman Coulter DTX 800/880 Series Multimode Detector (Beckman Coulter, Brea, CA, United States).

Quantification of Monoamines and Metabolites

Concentrations of NA, DA, 5-HT and their metabolites HVA, DOPAC and 5-HIAA were determined in the three brain regions using HPLC with electrochemical detection. This method has been described previously (Otten et al., 2010) and was used with a slightly adjusted extraction procedure. A 250-µl amount of brain samples homogenized in PBS was mixed on ice with 25 µl of 2 M and 25 µl of 0.2 M perchloric acid using a manual homogenizer. Following centrifugation (2,400 × *g*; 4°C; 10 min), the supernatant was preserved on ice while the pellet was resuspended in 300 µl of 0.2 M perchloric acid and centrifuged. The pooled supernatants were mixed and then centrifuged at 37,000 × *g* for 10 min at 4°C. The remaining pellet was weighed with a precision balance and the values obtained were used to calculate the concentration of neurotransmitters in each brain section. Aliquots of 40 µl were analyzed in duplicate. The HPLC system was equipped with a 125 mm × 4 mm reverse-phase column packed with Prontosil C18 AQ (Bischoff Analysentechnik, Leonberg, Germany). The mobile phase consisted of 58 mM sodium hydrogen phosphate buffer containing 1.2 mM octanesulfonic acid, 0.3 mM EDTA, 0.2 mM potassium chloride, and 9% methanol at pH 3.6, and was used at a flow rate of 1.2 ml/min. Electrochemical detection was achieved by a SenCell with a glassy carbon working electrode set at a potential of 600 mV (Axel Semrau GmbH, Sprockhövel, Germany). The HVA/DA, DOPAC/DA and 5-HIAA/5-HT ratios were calculated as an index of DA and 5-HT turnover.

RNA Isolation and cDNA Synthesis

Total RNA from previously homogenized brain samples was extracted using TRIzol Reagent (Life Technologies). RNeasy Mini Kit (Qiagen, Hilden, Germany) was used to purify the extracted product. The quality of the RNA was analyzed by horizontal electrophoresis on 1%-agarose gels, which validated the presence of intact 18S and 28S rRNA bands for the individual RNA specimens. The RNA concentration was determined with a NanoDrop One^{C} spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States). The RNA obtained was reverse-transcribed to single-strand cDNA using the SensiFAST cDNA Synthesis Kit (Bioline, London, United Kingdom). The reverse-transcriptase reaction was set at 42°C for 50 min with a subsequent inactivation step at 70°C for 15 min. Finally, the synthetized cDNA was diluted in 80 µl distilled water.

Real-Time Quantitative PCR (qPCR)

RNA samples from the brains of maraena whitefish exposed to the acute (sampled after 3 h or 24 h) and chronic handling experiments along with the respective controls were analyzed with multiplex qPCR technology (BioMark, Fluidigm, South San Francisco, CA, United States) to study the effects of handling on the transcript level. We designed a primer panel specific for the monoaminergic and neurological stress response. In this matter, orthologous gene sequences from Atlantic salmon *S. salar*, Coho salmon *Oncorhynchus kisutch*, and rainbow trout *O. mykiss* were aligned to the RNA-seq read collection of maraena whitefish *C. maraena* (Brietzke et al., 2016) using the program Bowtie2 (v 2.2.4). The resulting matching alignment with *C. maraena* was sorted and indexed with the Samtools package (v 1.6). Finally, the consensus sequences were visualized and redeemed using the software Ugene (v1.29). These sequences were used to design gene and species-specific primer pairs (see **Supplementary Table 1**) using the PSQ Assay Design Software 1.0.6 (Biotage AB, Uppsala, Sweden) for amplifying products with final lengths ranging from 140 to 180 bp. In addition, qPCR assays were carried out on 48.48-Dynamic Array IFC chips (Fluidigm) with EvaGreen fluorescence dyes (Bio-Rad, Hercules, CA, United States) using the BioMark HD-System (Fluidigm). One microliter of total RNA was reverse-transcribed using the Reverse Transcription Master Mix (Fluidigm). The resulting cDNA was adjusted to 10 ng/5 µl and underwent a subsequent pre-amplification of 11 cycles performed with the PreAmp Master Mix (Fluidigm) and the primers at a final concentration of 100 µM per primer pair. Afterward, preamplified cDNA was treated with exonuclease I (ExoI; New England BioLabs, Ipswich, MA, United States). Then, the cDNA samples were diluted in SsoFast EvaGreen Supermix with Low ROX (Bio-Rad) and the 20 × DNA Binding Dye Sample Loading Reagent. The cDNA samples and primer-pair mixes were transferred to the corresponding sample and assay inlets of the IFC chip using the IFC Controller RX (Fluidigm) and the 'Load Mix 48.48 GE' pre-set script. The IFC chip was transferred to the BioMark HD-System (Fluidigm) to perform the quantification reactions following the 'GE 48 × 48 Fast PCR + Melt v2.pcl' cycling program. The obtained qPCR data were analyzed using the Fluidigm RealTime PCR Analysis Software v.4.5.2. All qPCR products were run on a 2%-agarose gel to assess the integrity and specificity of the PCR products. An external standard was used to calculate the relative gene expression, which was normalized against the geometric mean of the copy numbers of *EEF1A1b*, *RPL9* and *RPL32* (Altmann et al., 2015). The 37 genes analyzed belonged to seven gene groups (**Table 1**). We studied a broad repertoire of genes involved in the synthesis (TH, TPH1, and TPH2) and degradation (MAO) of monoamines, and genes coding for monoamine receptors (ADR, DRD, and HTR) and markers for neuronal activity (FOSL) and neuronal plasticity (BDNF), downstream

TABLE 1 | Target genes.

	Official gene name; product	Function
Adrenergic receptors	ADRA1B; adrenoceptor α1B	Neuron signaling, regulation of transcription
	ADRA1D; adrenoceptor α1D	Neuron signaling, regulation of transcription
	ADRA2A; adrenoceptor α2A	Neuron signaling, regulation of transcription
	ADRA2B; adrenoceptor α2B	Neuron signaling, regulation of transcription
	ADRA2C; adrenoceptor α2C	Neuron signaling, regulation of transcription
	ADRA2D; adrenoceptor α2D	Neuron signaling, regulation of transcription
	ADRB2; adrenoceptor β2	Neuron signaling, regulation of transcription
	ADRB3A; adrenoceptor β3	Neuron signaling, regulation of transcription
Dopamine receptors	DRD1; dopamine receptor D1	Neuron signaling, regulation of transcription
	DRD2; dopamine receptor D2	Neuron signaling, regulation of transcription
	DRD3; dopamine receptor D3	Neuron signaling, regulation of transcription
	DRD4; dopamine receptor D4	Neuron signaling, regulation of transcription
	DRD5; dopamine receptor D5	Neuron signaling, regulation of transcription
5-HT receptors	HTR1A; 5-hydroxytryptamine receptor 1A	Neuron signaling, regulation of transcription
	HTR1B; 5-hydroxytryptamine receptor 1B	Neuron signaling, regulation of transcription
	HTR1D; 5-hydroxytryptamine receptor 1D	Neuron signaling, regulation of transcription
	HTR1E; 5-hydroxytryptamine receptor 1E	Neuron signaling, regulation of transcription
	HTR1F; 5-hydroxytryptamine receptor 1F	Neuron signaling, regulation of transcription
	HTR2A; 5-hydroxytryptamine receptor 2A	Neuron signaling, regulation of transcription
	HTR2B; 5-hydroxytryptamine receptor 2B	Neuron signaling, regulation of transcription
	HTR2C; 5-hydroxytryptamine receptor 2C	Neuron signaling, regulation of transcription
	HTR3A; 5-hydroxytryptamine receptor 3A	Neuron signaling
	HTR3C; 5-hydroxytryptamine receptor 3C	Neuron signaling
	HTR4; 5-hydroxytryptamine receptor 4	Neuron signaling, regulation of transcription
	HTR6; 5-hydroxytryptamine receptor 6	Neuron signaling, regulation of transcription
	HTR7; 5-hydroxytryptamine receptor 7	Neuron signaling, regulation of transcription
Monoamine synthesis and degradation	TPH1; tryptophan hydroxylase 1	Synthesis of 5-HT
	TPH2; tryptophan hydroxylase 2	Synthesis of 5-HT
	TH; tyrosine hydroxylase	Synthesis of catecholamines
	MAO; monoamine oxidase	Monoamine degradation
Neuronal activity	BDNF; brain derived neurotrophic factor	Growth factor, neurogenesis
	FOSL1; FOS Like 1, AP-1 transcription factor subunit	Transcription factor, neuronal activity
Microglia cell markers	CSF1R (MCSFR); Colony-stimulating-factor-1 receptor	Glia marker, cell differentiation
	MPEG1; Macrophage-expressed gene 1 protein	Glia marker, microbicidal activity
Cortisol receptors	NRC1a (GR1); glucocorticoid receptor 1	Regulation of transcription
	NRC1b (GR2); glucocorticoid receptor 2	Regulation of transcription
	NRC2 (MR); mineralocorticoid receptor	Regulation of transcription

Summary of the gene groups analyzed for differential expression in the brain of maraena whitefish. Official name, protein product and brief description of the function are indicated.

factors of the monoamine activation (Benito and Barco, 2015; Vindas et al., 2018). Cortisol receptor genes (GR1, GR2, and MR) were analyzed as feedback regulators of the monoamine system (Medeiros and McDonald, 2013). The expression of microglial cell markers (CSF1R and MPEG) was investigated as an indicator for stress effects on this immune cell population (Preston et al., 2018; Kuil et al., 2020).

Statistical Analysis

The effects of acute handling (fish sampled 3 and 24 h later) on the neurotransmitter concentration in the brain and cortisol in plasma were analyzed separately for statistical significance using one-way ANOVA and Dunnett's test for multiple comparisons. In this case, measurements of control groups from the 3 and 24 h experiments (n = 16 in total) were compared with the measurements of the fish at 3 h (n = 8) or 24 h (n = 8) after treatment. The control groups were also used to determine the basal level of neurotransmitters in the brain, which were analyzed using ANOVA followed by Tukey tests. For the 10-day chronic handling experiment, Student's t-test was used to compare the measurements of the control group (n = 8) with those of the treated group (n = 8) regarding neurotransmitter concentrations in the brain or cortisol levels in plasma. For the gene-expression analysis of the brain, t-test was used to compare the transcript level of each gene in the 3- and 24 h-acute-handling groups (n = 6 each) and the chronic-handling group (n = 6) with their corresponding control groups (n = 6 each). We reduced the sample size for gene expression to six

in each group to fit all groups onto the same Fluidigm chip for better comparability. The control groups from the acute handling experiment ($n = 12$) were used to determine the gene-expression profile of undisturbed fish between the different brain sections and within the gene groups. These were analyzed using the t-test. Tests were conducted using the software GraphPad Prism 8.0. We defined genes as being significantly expressed if they were up (>2-fold) or down-regulated (<−2-fold) with a p-value < 0.05.

RESULTS

Effects of Acute and Repeated Handling on Plasma Cortisol Concentration

The concentration of cortisol in plasma was analyzed for the acute (3 and 24 h post-challenge) and repeated handling experiments. In the acute handling experiment, plasma cortisol was significantly elevated (14.2 ± 3.1 ng/ml; $p < 0.05$) 3 h after handling compared to plasma concentrations in control fish (5.4 ± 0.7 ng/ml). At 24 h post-challenge, plasma cortisol (5.5 ± 0.8 ng/ml) did not differ significantly from control levels. After 10 days repeated handling, the cortisol concentration for the challenged fish (7.6 ± 2.3 ng/ml) showed no significant increase over the control fish (4.4 ± 0.5 ng/ml).

Basal Concentrations of Neurotransmitters and Acute-Handling Effects

We quantified the basal neurotransmitter concentrations in the different brain regions of undisturbed maraena whitefish (0 h; **Figures 3A–F**). The highest basal concentrations of neurotransmitters were found in the FB and MB while lowest were in the HB. The DA metabolites DOPAC and HVA were similarly distributed across the three brain regions at low concentrations (**Figures 3D,E**). The concentration of 5-HIAA showed a rostral-caudal gradient (**Figure 3F**).

The concentrations of monoamines and their metabolites in the different brain regions were measured at different times after a single episode of acute handling and compared to the baseline concentration of undisturbed fish (**Figure 3**). Brain neurochemistry was mainly affected by acute handling in the MB. The level of 5-HIAA significantly increased at 3 h post-handling in the MB compared to the undisturbed fish (**Figure 3F**). The three monoamine neurotransmitters NA, DA, and 5-HT significantly increased in the MB at 24 h after exposure to handling (**Figures 3A–C**). As the concentrations of metabolites DOPAC, HVA, 5-HIAA did not change (**Figures 3D–F**), the DOPAC/DA, HVA/DA, and 5-HIAA/5-HT ratios significantly decreased 24 h post-handling in the MB (**Figures 3G–I**) compared to the control group.

Expression Profiles of 37 Genes in Different Brain Regions, as Well as Acute-Handling Effects

We profiled the expression of 37 target and three reference genes (**Figure 4**) across the selected brain regions (**Figure 4A**) and compared the transcript levels according to their functional categories (**Figure 4B**). In the FB, the *ADR* and *HTR* genes were most strongly expressed (**Figure 4A**, gene groups 1 and 3). The 5-HT receptor gene *HTR6* was almost exclusively expressed in the FB and might thus be a suitable marker gene (**Figure 4A**, gene group 3). Besides this, *HTR4* and *HTR7* were strongly expressed in the FB compared to the MB (−2 to −12-fold) and the HB (−24 to −83-fold; **Figure 4A**, gene group 3). The *ADRA1d* and *DRD4* transcripts had a decreasing concentration gradient from rostral to caudal brain regions, similar to the 5-HT receptor genes *HTR1E, -1F, -2C, HTR4,* and *HTR7*. In contrast, the neurotrophin gene *BDNF* was highly expressed in the FB (**Figure 4A**, gene group 5), but less expressed in the MB and HB.

In the MB, *ADR,* and *HTR* expression was slightly lower than FB, while *DRD* genes shared similar expression levels in both FB and MB (**Figure 4A**, gene groups 1–3). *HTR3A* was specifically expressed in the MB and it may be a potential marker gene for this brain region. Similar to *HTR3A*, *HTR1D* was slightly stronger expressed in the MB than in the other brain regions. In contrast, there was less expression of the *ADRA1b* and *ADRA2c* genes in the MB (**Figure 3A**, gene group 1). The expression of *ADR* and *DRD* genes was markedly lower in the HB than in the other sections, especially for *ADRA2a* and *ADRA2d* (**Figure 4A**, gene group 1). In contrast, *ADRA1b* and *ADRA2c* were strongly expressed in the whole brain of maraena whitefish, while the *ADRB* genes were least strongly expressed (**Figure 4B**, gene group 1). Among dopaminergic receptor genes, *DRD1* was most strongly expressed in the brain compared to the other *DRD* genes, which shared similar (though lower) transcript levels (**Figure 4B**, gene group 2). Among the serotonergic receptor genes, *HTR1A*, *-2B* and *-3C* were the most widely expressed throughout the brain with no differences between brain sections (**Figure 4B**, gene group 3).

The microglial markers *CSF1R* and *MPEG* were evenly distributed across the three brain sections (**Figure 4A**, gene group 6), with *CSF1R* being expressed more than ten times as much as *MPEG* (**Figure 4B**, gene group 6). In the three brain sections, expression of *GR2* and *MR* was two to three times higher than *GR1* (**Figure 4B**, gene group 7). Among the genes coding for the tryptophan hydroxylase (Tph) enzymes, *TPH1* was slightly less expressed in HB than in FB, while *TPH2* was more strongly expressed in MB and HB than in FB. Regarding the expression of both *TPH* genes, *TPH1* was more strongly expressed in the FB and MB than *TPH2* (**Figures 4A,B**, gene group 4).

Acute handling affected the expression of genes coding for the adrenergic receptor subtypes *ADRA1* and *ADRB* in the HB region (**Figure 5**, gene group 1). In particular, *ADRA1d* (−2.2-fold) and *ADRB3* (−2.5-fold) were down-regulated 3 h after handling. Genes coding for the different subtypes of serotonergic receptors were also affected by acute handling throughout the brain (**Figure 5**, gene group 3). *HTR1D* transcript level increased 6.3-fold after 3 h in the HB and 2.8-fold after 24 h in the FB. *HTR6* was up-regulated (3.5-fold) in the MB 3 h post-handling. *HTR1A* and *HTR3C* were down-regulated (−2-fold) after acute-handling in the HB, while *HTR3C* was also down-regulated (−2.9-fold) in the FB after 24 h.

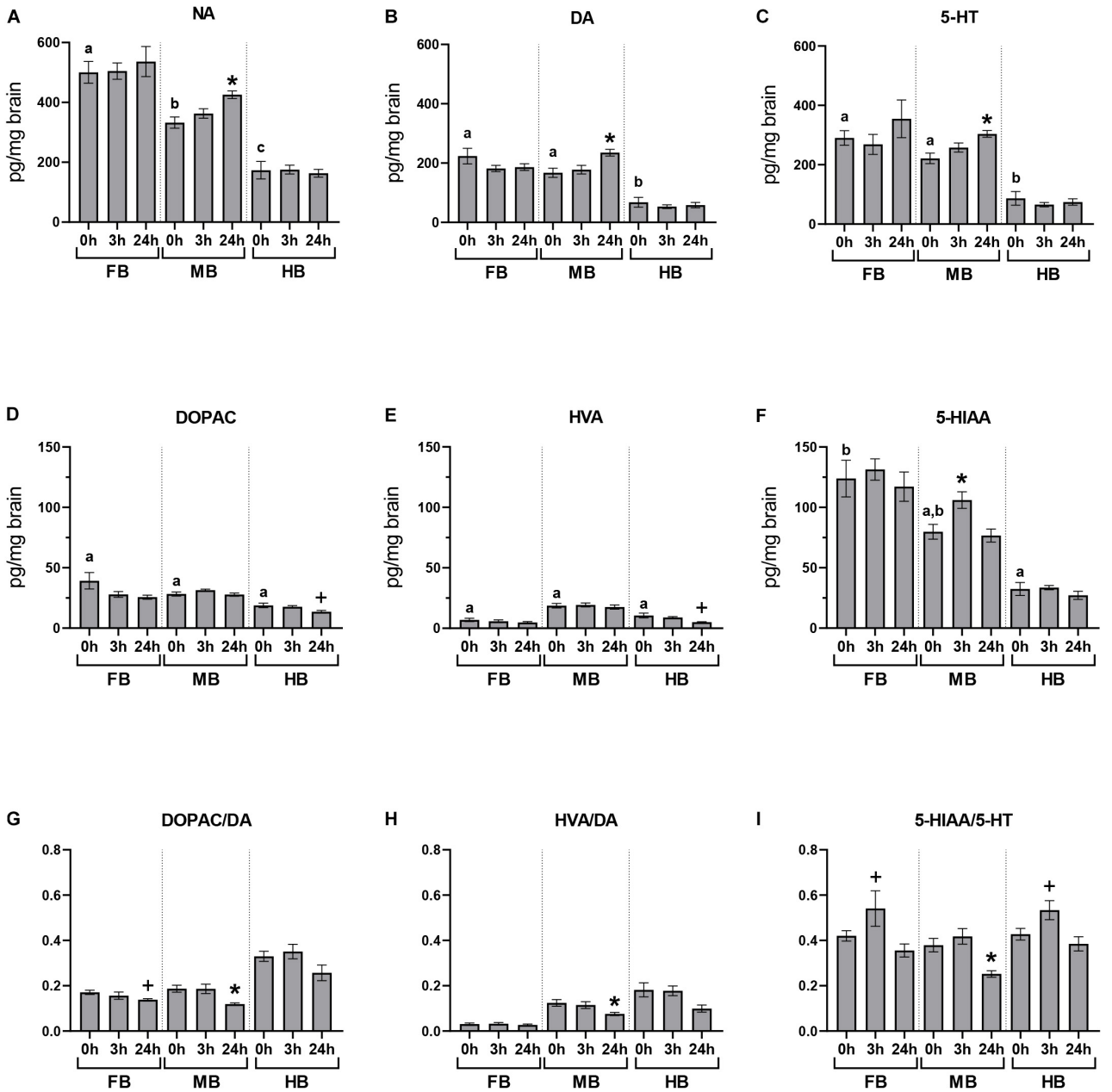


FIGURE 3 | Acute handling effects on neurotransmitter concentrations in the brain of maraena whitefish. The effects of acute stress were analyzed section-wise at 0 h (n = 16), 3 h (n = 8), and 24 h (n = 8) after exposure to acute handling for **(A)** NA; **(B)** DA; **(C)** 5-HT; **(D)** DOPAC; **(E)** HVA; **(F)** 5-HIAA concentrations (pg/mg tissue); and the ratios of **(G)** DOPAC/DA; **(H)** HVA/DA; and **(I)** HIAA/5-HT. Comparisons between handling and control groups were tested for statistical significance using one-way ANOVA and Dunnett's test for multiple comparisons and is indicated by (*) at $p < 0.05$ or non-significant tendency indicated as (+) at $0.05 < p < 0.10$. The controls of the acute stress experiments (0 h; n = 16) were used to determine the abundance and distribution of neurotransmitters and metabolites in the three brain sections of undisturbed maraena whitefish. Statistical analysis was performed using ANOVA with the Tukey test ($p < 0.05$). Error bars indicate SEM; different characters **(a–c)** above the bars indicate statistically significant concentration differences between brain regions and within time points.

Effects of Repeated Handling on Brain Neurotransmitter Concentrations and Gene Expression

After the 10-day period of daily handling, brain neurotransmitters were analyzed 24 h after the previous handling episode (**Figure 6**). NA concentration was slightly increased in FB and MB after 10 days of repeated handling ($p < 0.10$; **Figure 6A**), but the concentration of the other monoamines and metabolites was unaffected.

The expression of dopaminergic receptor genes was modulated in the FB and MB after 10 days of repeated handling (**Figure 5**, gene group 2). *DRD1* was −2.0-fold down-regulated

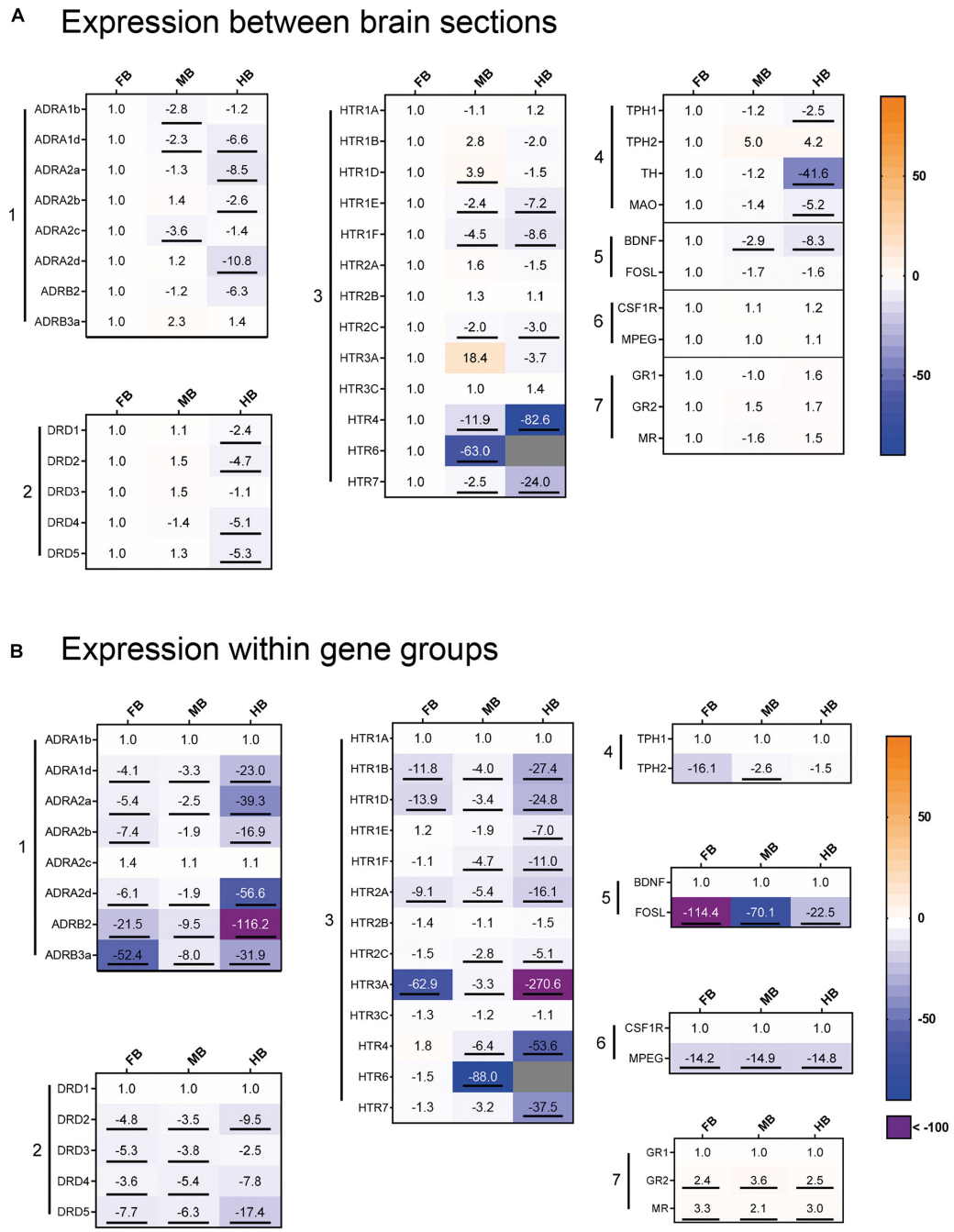


FIGURE 4 | Gene expression profile of maraena whitefish brain. The gene expression was profiled in the control animals of the acute stress experiment (3 and 24 h; $n = 12$) and analyzed with regard to **(A)** the different brain sections and **(B)** to the gene groups expressed. Results are expressed as fold-change values and colored according to the legend on the right. **(A)** To assess the gene expression in the different sections, the gene-specific transcript numbers in the MB and HB are shown relative to the respective transcript levels in the FB, which were set at 1.0. **(B)** Within the gene groups, the transcript numbers of the different genes are shown relative to the respective transcript levels of the gene in the first row, which were set at 1.0. The gene groups comprised (1) adrenergic receptor genes; (2) dopamine receptor genes; (3) 5-HT receptor genes; (4) monoamine synthesis genes; (5) neuronal activity markers; (6) microglia cell markers; (7) cortisol receptor genes. Genes were considered significantly regulated (marked by an underline) in the event of at least a twofold up or down-regulation of the transcript concentration and a p-value < 0.05 (Student's t-test).

in the FB and *DRD4* was −2.9-fold down-regulated in the MB. In addition, the transcript levels of the enzymes involved in monoamine synthesis were modulated in the FB and HB of maraena whitefish. *TPH1* was −3.6-fold down-regulated in the FB, while *TH* was 3.3-fold up-regulated in the HB (**Figure 5**, gene group 4).

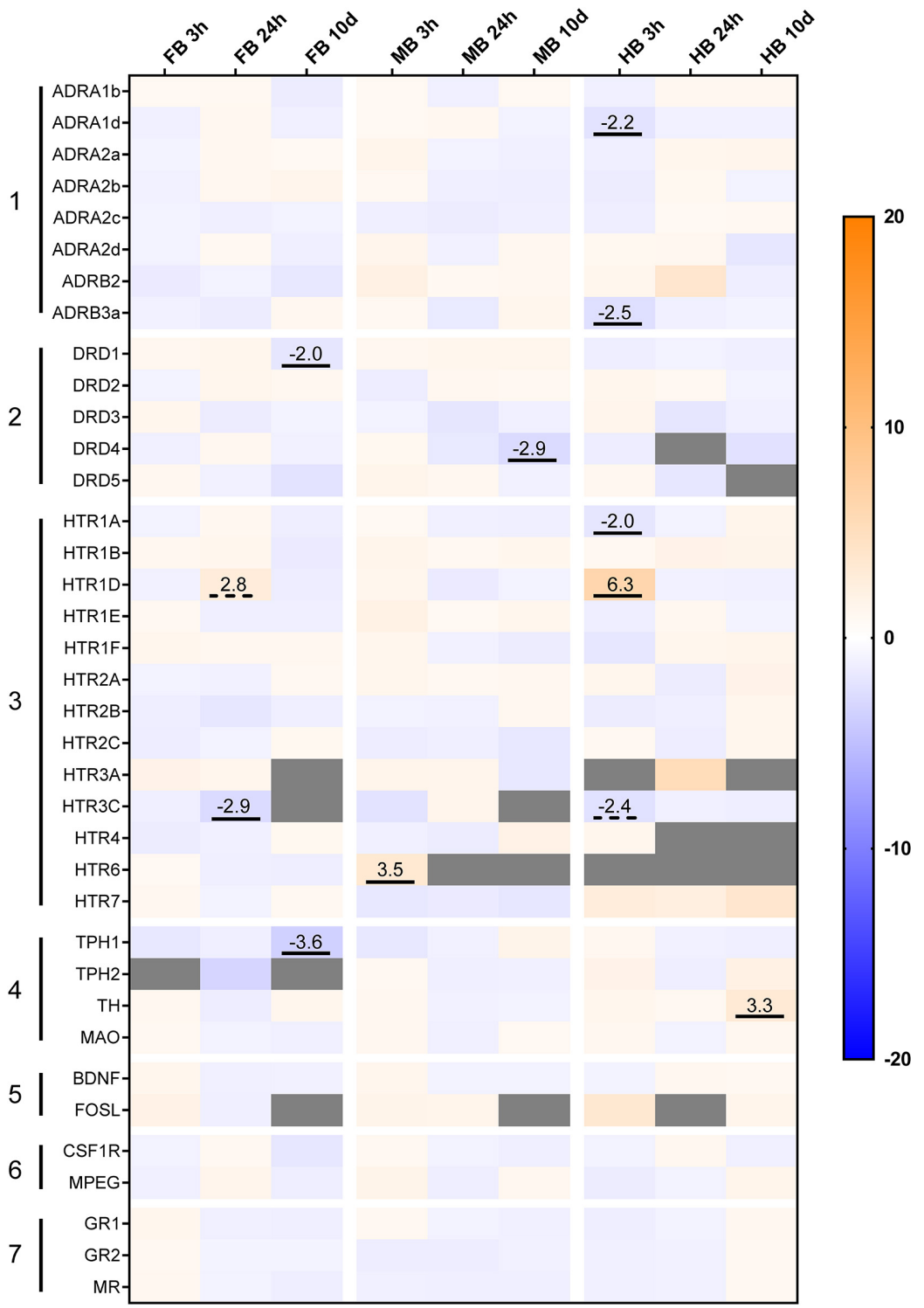


FIGURE 5 | Heatmap illustrating the effects of acute and chronic handling on gene expression in the brain of maraena whitefish. The heatmap shows the gene expression in fold-change values in the different brain sections after acute (3 and 24 h) and chronic (10 days) handling stress. The transcript level of each gene (n = 6) in the different brain sections and time-points was compared to its corresponding control group (n = 6 each). The scale bar indicates up-regulation (orange) or down-regulation (blue). Gray cells indicate non-detectable expression. The gene groups comprised (1) adrenergic receptor genes; (2) dopamine receptor genes; (3) 5-HT receptor genes; (4) monoamine synthesis genes; (5) neuronal activity markers; (6) microglial markers; (7) cortisol receptor genes. Genes were considered significantly regulated in the event of either an (at least) twofold up- or down-regulation of the transcript concentration and a p-value < 0.05 (marked by an underline) or non-significant tendencies at $0.05 < p < 0.10$ (are marked with dashed lines).

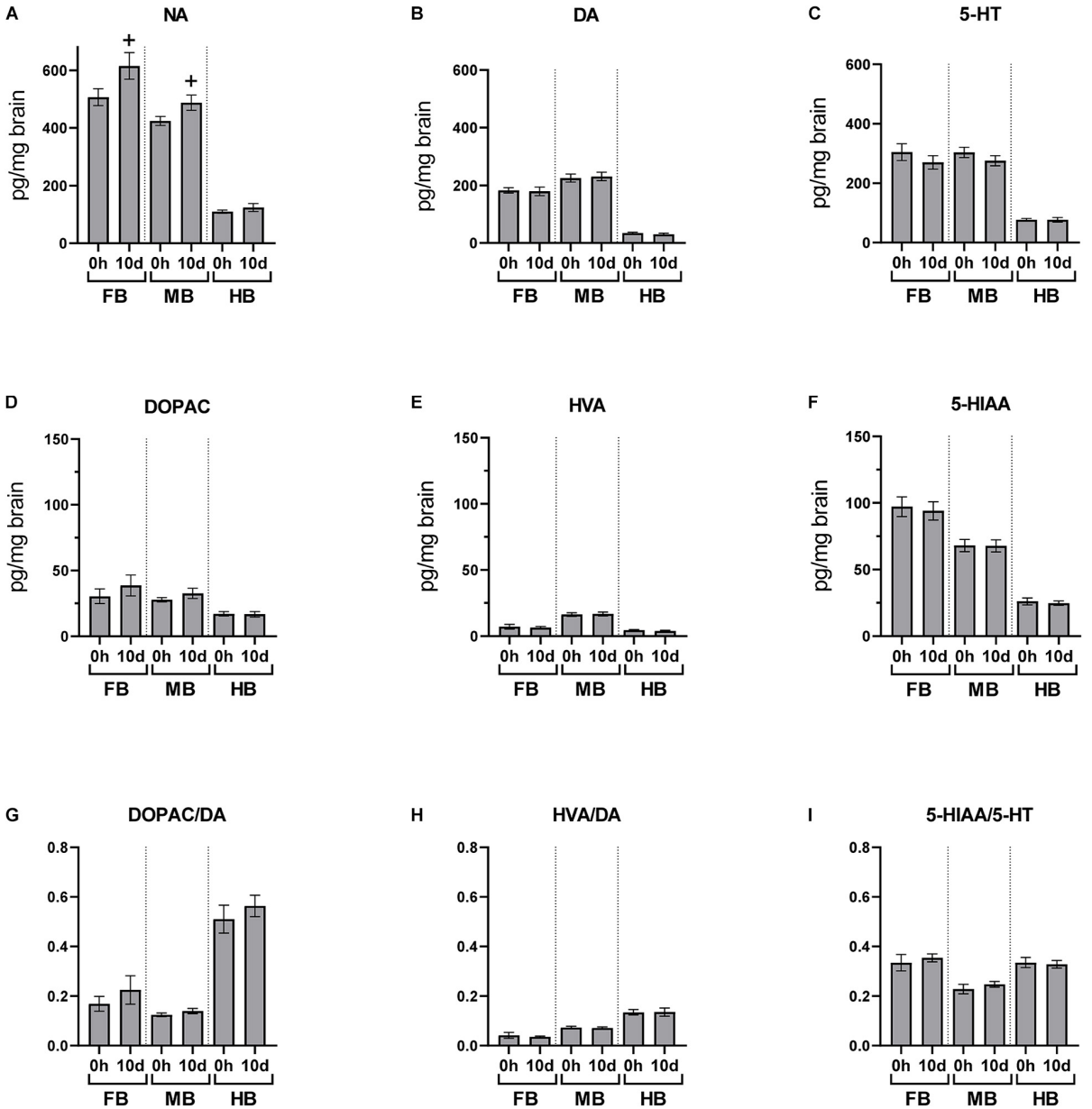


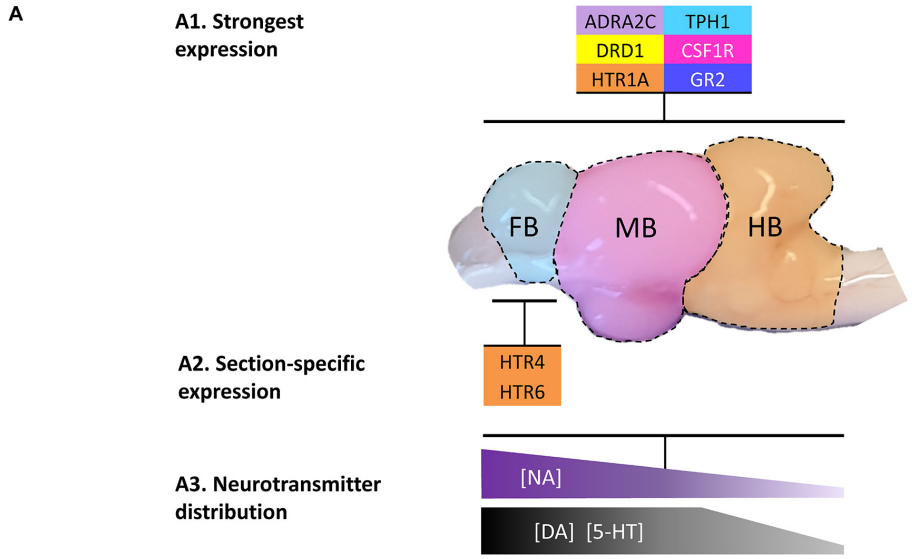
FIGURE 6 | Chronic handling effects on the neurotransmitter concentrations in the brain of maraena whitefish. The effects of repeated handling were analyzed section-wise at 0 h (n = 16) and 10 days (n = 8) after exposure to acute handling for **(A)** NA; **(B)** DA; **(C)** 5-HT; **(D)** DOPAC; **(E)** HVA; **(F)** HIAA concentrations (pg/mg tissue); and the ratios of **(G)** DOPAC/DA; **(H)** HVA/DA; and **(I)** HIAA/5-HT. Statistical significance was assessed with Student's t-test; non-significant tendency is indicated as (+) at $0.05 < p < 0.10$.

DISCUSSION

Low Monoamine Activity in the Hindbrain and Specific Gene Expression Patterns in Brain Sections of Maraena Whitefish

The first part of this study investigated the gene expression and neurochemistry of sixteen individual maraena whitefish, which served as reference individuals in the present study. Although this cohort was considered as "control fish," it was exposed to unavoidable challenges, i.e., husbandry in the anthropogenic environment as well as the sampling procedure.

Monoamine concentrations were analyzed in the three selected sections of the brain (**Figure 7**). We observed a decreasing concentration gradient of the investigated neurotransmitters from FB to HB (**Figure 7A**). This is consistent with our observation that the genes coding for monoamine receptors are expressed more strongly in FB and MB and less strongly in HB (**Figure 4A**; gene groups 1–3). This finding may indicate a lower influence of monoamines



B

		FB		MB		HB	
ACUTE 3h	NTs	↑5-HIAA/5-HT⁺	----	↑5-HIAA*	----	↑5-HIAA/5-HT⁺	----
	Genes	----	----	↑HTR6*	----	↑HTR1D*	↓ADRA1D* ↓ADRB3a* ↓HTR1A* ↓HTR3C⁺
ACUTE 24h	NTs	----	↓DOPAC/DA⁺	↑NA* ↑DA* ↑5-HT*	↓HVA/DA* ↓5-HIAA/5-HT* ↓DOPAC/DA*	----	↓DOPAC⁺ ↓HVA⁺
	Genes	↑HTR1D⁺	↓HTR3C*	----	----	----	----
CHRONIC 10d	NTs	↑NA⁺	----	↑NA⁺	----	----	----
	Genes	----	↓DRD1* ↓TPH1*	----	↓DRD4*	↑TH*	----

FIGURE 7 | Graphical summary about **(A1)** the most strongly expressed genes with a similar distribution across the three brain sections; **(A2)** those genes that were particularly high expressed in specific sections; and **(A3)** the spatial distribution of specific neurotransmitters. **(B)** Overview of the parameters that were affected by acute and chronic handling at the different time points in the analyzed brain sections. (↑) increased or (↓) decreased ratios/concentrations in case of neurotransmitters (NT) and >2-fold up- or <–2-fold down-regulation in the case of gene expression. Statistical significance is indicated with (*) at $p < 0.05$; non-significant tendency indicated as (+) at $0.05 < p < 0.10$. See **Figure 5** for heatmap with all target genes.

on HB. Higher concentrations of the monoamines investigated have already been described in the telencephalon and midbrain of the client reef fish *Labroides dimidiatus* and *Naso elegans* (Abreu et al., 2018), rainbow trout *O. mykiss* (Øverli et al., 2001) and Arctic charr *S. alpinus* (Backström et al., 2017). The high basal expression of *ADR* and *HTR* genes in the FB compared to other parts of the brain suggests that the telencephalon is the main target of 5-HT and NA. This assumption was supported by the high level of stored NA in this section, which may indicate the strong innervation of these areas by noradrenergic neurons. The high level of the metabolite 5-HIAA in the FB is most likely the result of 5-HT metabolization processes. The high concentration of monoamines and metabolites in the FB is coinciding with the high expression of *MAO*, which is essential for the degradation of monoamines (Winberg and Nilsson, 1993).

In our study, the expression of *DRD* genes in the HB was generally lower than in the other sections. *DRD1* was the most strongly expressed dopaminergic receptor gene in the three brain sections analyzed in maraena whitefish. In contrast to our findings, *DRD1* of zebrafish *D. rerio* was mainly found in the brain stem and hypothalamic region, but not in the forebrain (Maximino and Herculano, 2010). We found that the expression of *HTR* genes was particularly high in the FB of maraena whitefish compared to the other sections. In humans, prefrontal cortex and hippocampus express almost all *HTR*-encoding genes, which are a main target of serotonergic innervation (Švob Štrac et al., 2016). The corresponding regions of fish are presumably located

in the telencephalic area and include the equivalent to the amygdala, POA or the *striatum*, which are involved in the stress response and subjected to 5-HT signaling. Among the HTR genes analyzed, *HTR1A* was the most abundant transcript, which was present in all three brain sections. A similar observation has been made in Gulf toadfish *Opsanus beta* where 5-HT-1A modulates the HPI axis (Medeiros et al., 2010). In mammals, 5-HT-1A is also widely distributed in the brain, mainly present in the limbic areas and the raphe nuclei where it inhibits the 5-HT signaling and modulates the HPA axis (Fuller, 1992; Barnes and Sharp, 1999).

HTR4 and especially *HTR6* were highly expressed in the telencephalon compared to the rest of the brain (**Figure 7A**). In mammals, these 5-HT receptors act post-synaptically and induce cAMP signaling upon 5-HT binding (Branchek and Blackburn, 2000; Rosel et al., 2004). A high *HTR4* expression has been observed in progenitors of motor neurons in zebrafish *D. rerio* (Barreiro-Iglesias et al., 2015). In line with our results, mammalian *HTR4* is abundantly present in the mammalian nigrostriatal system that corresponds in teleosts to a region from the diencephalon to the telencephalon (**Figure 1**) (Barnes and Sharp, 1999; O'Connell et al., 2011). The expression pattern of *HTR6* in the telencephalon of maraena whitefish is similar to that in the brain of the cichlid *Astatotilapia burtoni* (Loveland et al., 2014). Previous studies on zebrafish (Lillesaar, 2011; Gaspar and Lillesaar, 2012) suggest that 5-HT populations express *TPH1* particularly in the hypothalamus (MB in this study), and *TPH2* in the pretectal complex and raphe (MB and HB in this study). Our finding of similar expression levels of *TPH1* in FB and MB of maraena whitefish was unexpected because 5-HT neurons, which normally express this gene, have not been described in telencephalon so far. We could speculate that *TPH1* is expressed by axonal projections of hypothalamic 5-HT populations into telencephalic regions similar to the expression of *TPH2* by neurons from the *raphe nuclei* (Lillesaar et al., 2009). Alternatively, we may have included in the dissection of the FB the pineal gland or part of the posterior *tuberculum*, which have been described to contain *TPH1*-expressing neurons (Gaspar and Lillesaar, 2012).

The LC is situated in the brainstem of teleosts and harbors an important noradrenergic cell population (Ekström et al., 1986). Therefore, the presence of *TH* and the synthesis of catecholamines would be expected here (Kaslin and Panula, 2001). However, *TH* transcripts were almost absent in HB. Concentrations of NA, as well as DA and its metabolites, were also low. The relatively small size of the LC in relation to the HB section might be an explanation for this finding.

Cortisol is the end product of the HPI axis and a strong regulator of the monoaminergic response in the brain (Medeiros and McDonald, 2013). The expression of the cortisol receptor genes *GR2* and *MR* was significantly higher throughout the brain than that of *GR1*, which suggests that these receptors might play a stronger role in cortisol signaling in the brain, compared to other organs where GR1 is predominant (Martorell-Ribera et al., 2020).

The expression of *BDNF*, which is an important factor for neurogenesis and cell proliferation, was particularly high in the FB. The brain of the killifish *Nothobranchius furzeri* displayed a similarly pronounced expression of *BDNF* in the dorsal telencephalic areas, suggesting that this region is essential for neuronal growth and plasticity (D'Angelo et al., 2014).

Acute Handling Increased 5-HT Turnover and Modulated Receptor Expression at 3 h Post-handling

Brain neurochemistry responded to acute handling with significantly increased 5-HIAA in the MB at 3 h post-handling, together with a slightly increased 5-HT turnover in FB and HB. We speculate that this is the result of an elevated release of 5-HT during and immediately after handling. A previous study demonstrated an increased 5-HT turnover in the telencephalon and hypothalamus of rainbow trout induced by chasing (Gesto et al., 2013). In addition, increased 5-HIAA concentrations have been linked to isolation and confinement stress of rainbow trout (Øverli et al., 2001). In Arctic charr, 5-HT and 5-HIAA concentrations in the brain stem of dominant fish were lower than in subordinate fish (Backström et al., 2015). In our study, serotonergic activation was accompanied by significantly elevated plasma cortisol levels 3 h after treatment, which indicates an activated HPI axis (Wendelaar Bonga, 1997). Although this cortisol concentration was significantly higher than that of control fish, it was still below the established stress levels of rainbow trout (Iwama et al., 1999; Barton, 2000; Schreck et al., 2016). This may be an indication that the cortisol concentration may have been higher before our first sampling, while it was already decreasing at the time of sampling. Acute handling up-regulated the *HTR6* expression in the MB of maraena whitefish. The basal expression of *HTR6* in the MB is extremely low and this condition might indicate its rather subordinate role in the response to handling, especially in view of the high *HTR6* expression in the FB of undisturbed fish.

The HB of maraena whitefish was expected to have important 5-HT neuron populations, which have been found in the NLV and *raphe nuclei* of several teleost species (Lillesaar, 2011). However, lower basal concentrations of 5-HT and 5-HIAA were observed in the HB compared to the other sections. This section displayed weakly increased 5-HT turnover 3 h after handling (**Figure 7B**). Contrary, acute stressors have been reported to increase of 5-HIAA/5-HT ratios in the brain stem of rainbow trout (Gesto et al., 2008).

Our results showed that the expression of the *HTR1* family genes was affected 3 h after handling. *HTR1D* was up-regulated, while *HTR1A* was downregulated. In mice, 5-HT-1D regulates the activity of 5-HT neurons in the *raphe nuclei* (Vogelgesang et al., 2017) by inhibiting 5-HT actions (Huang and Thathiah, 2015). 5-HT-1A controls the HPA axis in mammals and also inhibits 5-HT signaling and, at the same time, it is regulated by a negative feedback after cortisol release (Fuller, 1992; Zhong and Ciaranello, 1995; Barnes and Sharp, 1999). In rainbow trout, *HTR1A* was down-regulated in the telencephalon 1 to 4 h post-stress (Moltesen et al., 2016), while it was upregulated in subordinate Atlantic salmon parr (Thörnqvist et al., 2015).

5-HT-3C promotes 5-HT signaling in mammals (Sangkuhl et al., 2009). In this study, *HTR3C* was significantly down-regulated in the brain of maraena whitefish 24 h post-handling

(**Figure 7B**). The up-regulation of *HTR1D* and down-regulation of *HTR3C* are most likely compensatory responses to counteract this 5-HT release, which could affect the *raphe* 5-HT populations of the brain stem, as described in brook trout *Salvelinus fontinalis* and other teleosts (Bolliet and Ali, 1992; Lillesaar, 2011). The adrenergic receptor genes *ADRA1D* and *ADRB3a* were also down-regulated in the HB 3 h post-handling. Both adrenergic receptors act post-synaptically as activating modulators of NA actions in the target neuron (Graybiel and Penney, 1999; Huang and Thathiah, 2015; Maletic et al., 2017). *ADRA1D* has been shown to be up-regulated in the hippocampus of rats under restraint stress (Campeau et al., 2010). *ADRB3* genes code for β_3 receptors, which are generally involved in NA release and neuron plasticity and are down-regulated during chronic stress in mammals (Seki et al., 2018). Although no changes in NA brain levels were observed 3 h post-handling, the down-regulation of these two genes suggests that acute handling might result in desensitization to NA in the HB.

The Initial Response to Handling Was Compensated by Increased Monoamine Concentrations at 24 h Post-handling

The initial serotonergic response 3 h post-handling was followed after 24 h by increased concentrations of NA, DA and 5-HT in the MB compared to control fish (**Figure 7B**). We assume that the increased monoamine concentrations are the compensatory response to the stress-related demand of monoamines to cope with stress. The observed increase in neurotransmitters could be explained by a reduced monoamine metabolism. However, the metabolites were not reduced compared to the value at 0 h and the gene expression of *MAO* was unaffected by stress. Alternatively, the elevated monoamine concentrations could be the result of an increased synthesis as a consequence of an accelerated enzymatic activity of TPH and TH or the increased availability of their substrates tryptophan and tyrosine. Both enzymatic activity and substrate availability are likely to be affected by stress as previously shown (Dunn and Welch, 1991; Chen and Miller, 2012). However, we did not detect an increased *TPH* and *TH* gene expression at sampling times of 3 and 24 h. Unfortunately, our data do not permit a final conclusion because we have neither analyzed the protein content expression nor the activity of the relevant enzymes. We think that the unchanged metabolite concentrations and return of plasma cortisol levels to baseline 24 h after the challenge reflect recovery from stress and a return of HPI activity to baseline between 3 and 24 h after the challenge. In teleost fish, low reactivity to stress may indicate an increased allostatic load (Madaro et al., 2015; Moltesen et al., 2016), which also impairs the natural stress-response mechanisms (Schreck et al., 2016). This implies the stress-dependent activation of the DA and NA systems, although we did not detect any significantly altered levels of DA or NA in the brain of maraena whitefish 3 h post-handling. After 24 h post-handling, only the expression of 5-HT-related genes in the FB was affected (**Figure 7B**). The down-regulation of *HTR3C*, which activates 5-HT signaling, and the up-regulation of *HTR1D*, which antagonizes the 5-HT signaling, suggest a balanced 5-HT activity that migrated from the rear brain at 3 h post-handling to the telencephalon 24 h post-handling.

Repeated Handling Evoked a Weak Monoaminergic Response

The 10-day repeated handling had no significant effects on the brain neurochemistry (**Figure 7B**). Although the fish in the chronic experiment were sampled 24 h after the last treatment, the monoaminergic activity was not consistent with the observed recovery period 24 h after acute treatment. Explanations for these observations could be habituation processes, exhaustion of neural responses or the effects of chronically elevated cortisol levels on the monoaminergic systems. The possibility that the decreased neurochemical response could be due to continuously elevated cortisol levels in the brain could be excluded, since the cortisol levels of repeatedly handled fish did not differ from those of control fish at the end of the 10-day experiment and were not significantly elevated even 24 h after acute handling. Moreover, the observed plasma cortisol levels remained below the stress levels described for rainbow trout (Schreck et al., 2016). Increased DA metabolite concentrations without altered plasma cortisol levels have been previously described in Arctic charr in response to handling (Backström et al., 2017). Depletion or exhaustion of neuronal responses are not very likely as we did not observe a reduction of the neurotransmitter pool compared with controls, which might decrease the monoaminergic response. Instead, we found subtle modulations of the *TPH1* and *TH* expression. This suggests that the monoamine synthesis pathways were sensitive to repeated handling and may indicate habituation.

Isolated rainbow trout had increased NA levels in the telencephalon and the optic tectum after 1 week (Øverli et al., 1999, 2001). Our results are consistent with these reports, since we also observed slightly increased concentrations of noradrenaline together with an upregulation of *TH* in HB.

Previous studies on the POA in the brain of cichlid fish revealed increased levels of tyrosine hydroxylase after handling stress, and a modulated expression of dopamine receptors and genes related to 5-HT synthesis after repeated handling (Chabbi and Ganesh, 2015).

DRD1 and *DRD4* were down-regulated in the FB and MB of maraena whitefish, respectively. D1 activates the dopamine signaling, while D4 inhibits it (Svingos et al., 2000; Centonze et al., 2003; Mizuno et al., 2007). In studies with rodents, acute and chronic stress modulated the expression of *DRD1*, depending on the forebrain region analyzed (Rasheed et al., 2012). The DA receptor D4 participates in the dopaminergic stress response of primates (Arnsten et al., 2000). Taking into account the aforementioned studies, the down-regulation of *DRD1* and *DRD4* in our results points to the desensitization of the DA system in the FB and MB.

Our results suggest that the 5-HT system was desensitized by a repeated stressor compared to the 5-HT response to a single episode of handling (see sections "Acute Handling Increased 5-HT Turnover and Modulated Receptor Expression at 3 h Post-handling" and "The Initial Response to Handling was Compensated by Increased Monoamine Concentrations at 24 h

Post-handling"). However, markers derived from the synthesis pathway of 5-HT are apparently promising indicators of the serotonergic response to chronic stressors. TPH controls the rate-limiting step of synthetizing 5-HT from tryptophan. Recently, a study demonstrated a downregulation of *TPH1* and *TPH2* after heat stress in medaka fish *Oryzias latipes* (Shimomura et al., 2019). In our experiment, the repeated handling reduced *TPH1* expression in the FB section of maraena whitefish. However, as discussed in Section "Low Monoamine Activity in the Hindbrain and Specific Gene Expression Patterns in Brain," the expression of *TPH1* was detected in 5-HT neurons, which should not be present in the brain parts included in our FB sections. Therefore, further investigations based on precise histology are required to confirm our data.

CONCLUSION

The effects of stress on the brain neurochemistry vary remarkably throughout the numerous interrelated regions of the brain. In this study, we observed that acute handling activates the HPI axis and serotonergic activity of the brain shortly after the challenge. At 24 h after acute handling, the return of plasma cortisol to baseline together with an increase of brain monoamine concentrations indicated a recovery. After 10 days of repeated handling, the modest neurochemical response and the low cortisol levels might reflect a habituation to the persistent challenge, as the increase of the NA concentration is much weaker than at 24 h after the single handling event. Based on the basal expression and the significant regulation under handling conditions, we selected a group of genes as potential markers that indicated the effects of handling as a stressor on the brain monoamine systems, i.e., *DRD1*, *DRD4*, *ADRA1D*, *ADRB3a*, *HTR1A*, and *HTR3C*. Our results suggest that maraena whitefish should be left undisturbed for at least 24 h following routine aquaculture procedures that include intense handling, such as size sorting or transportation. This salmonid species apparently habituates to repeated handling in the long term. Our study shows that a stress-sensitive species such as maraena whitefish is able to adapt to the anthropogenic stressors related to aquaculture conditions if enough time is made available between challenges.

DATA AVAILABILITY STATEMENT

Datasets generated for this study are available from the corresponding authors on request.

ETHICS STATEMENT

The animal study was reviewed and approved by Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg-Vorpommern, Germany (LALLF M-V/TSD/7221.3-1-069/18).

AUTHOR CONTRIBUTIONS

UG, AR, JM-R, and TG designed the research. UG, AR, and TG supervised the experiments. RB organized the husbandry of the maraena whitefish. JM-R and MV performed stress experiments and sampled fish. JM-R and WO performed HPLC analyses. JM-R and AR conducted RT-qPCR analyses. JM-R performed statistical analyses. JM-R wrote the manuscript. AR and UG edited the manuscript. All the authors have read and agreed to the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2020.591738/full#supplementary-material

Supplementary Table 1 | Target genes and primers designed for RT-qPCR analysis.

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6. Summary

Polysialic acids (polySia) are carbohydrate polymers, well known for their repulsive properties and essential functions during brain development. Alterations in the polysialylation machinery are associated with several diseases in mammals. In contrast to mammals, fish generate a wide range of different polySia structures. However, little is known about the occurrences and the functions of polySia on fish.

In the outlined dissertation, I investigated the distribution of the polysialyltransferases (polySTs) ST8Sia II and ST8Sia IV, which are responsible for polySia biosynthesis in fish. Using bioinformatics strategies, the evolutionary history of polySTs was reconstructed in fish genomes. This teleost phylogenetic study revealed that in contrast to mammals, the fish polySTs appear to be much more diverse and show a patchy distribution. This includes duplications or losses of the different *st8sia* genes. For instance, the Salmonidae genome contained three polySTs (*st8sia2-r1*/*st8sia2-r2* and *st8sia4*) in contrast to one (*st8sia2*) in Percidae. The expression profiling of the polySTs in Salmonidae (*Coregonus maraena*) suggest that in ovaries a synthesis of polySia occurs, which was confirmed by Western blotting against polySia. Based on this, we determine the polySia-positive cells by immunohistochemistry in the ovaries. The obtained results demonstrate that clusters of oogonia are the main source of polySia, which was also observed in Percidae (*Sander lucioperca*), which lost *st8sia4* during evolution. In line with that, *st8sia2-r2* was the dominant polyST in Salmonidae. Since polySia seems to be regulated during acute stress in mice, a stress handling study has been done in *Coregonus maraena*. However, no significant changes in the expression levels of polySTs were detected.

In summary, the obtained results suggest that polySia is differently used than in mammals.

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Declaration of contribution in each publication

I hereby declare that my contribution in each publication summarized in this dissertation is as follows:

Study I

Vertebrate Alpha2,8-Sialyltransferases (ST8Sia): A Teleost Perspective

Venuto, M.T.; Decloquement, M.; Martorell Ribera, J.; Noel, M.; Rebl, A.; Cogez, V.; Petit, D.; Galuska, S.P.; Harduin-Lepers, A. *Int. J. Mol. Sci.* **2020**, *21*, 513. (https://doi.org/10.3390/ijms21020513)

- Literature research and writing the article
- Investigation and methodology
- Database research
- Phylogenetic analysis
- Synteny/paralogy analysis
- Sequence alignments
- 3D structure construction
- tissues collection and processing
- Discussion and interpretation of the data
- Writing – original draft preparation

Study II

Characterization of the Polysialylation Status in Ovaries of the Salmonid Fish Coregonus maraena and the Percid Fish Sander lucioperca

Venuto, M.T.; Martorell-Ribera, J.; Bochert R.; Harduin-Lepers A.; Rebl A.; Galuska SP.; *Cells*. 2020; 9(11):2391. (https://doi.org/10.3390/cells9112391)

- Ovary collection and processing
- WB experiment

- Immunohistochemistry experiments
- Phylogenetic analysis
- Synteny analysis
- Statistical evaluation of the data
- Discussion and interpretation of the data
- Writing – original draft preparation

Study III

Time-Dependent Effects of Acute Handling on the Brain Monoamine System of the Salmonid Coregonus maraena

Martorell-Ribera J.; **Venuto M.T.**; Otten W.; Brunner R.M.; Goldammer T.; Rebl A.; Gimsa U.; Front. 2020; 14. (https://doi.org/10.3389/fnins.2020.591738)

- Stress experiment
- Sample collection and processing

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Conference and publication contributions

Publications

Peer-reviewed works (*part of the doctoral thesis)

1. ***Venuto, M.T.**, Decloquement, M., Martorell-Ribera, J., Noel, M., Rebl, A., Cogez, V., Petit, D., Galuska, S.P., & Harduin-Lepers, A. (2020). Vertebrate Alpha2, 8-Sialyltransferases (ST8Sia): A Teleost Perspective. International journal of molecular sciences, 21(2), 513. https://doi.org/10.3390/ijms21020513
2. ***Venuto, M.T**., Martorell-Ribera, J., Bochert, R., Harduin-Lepers, A., Rebl, A., & Galuska, S.P. (2020). Characterization of the Polysialylation Status in Ovaries of the Salmonid Fish *Coregonus maraena* and the Percid Fish Sander lucioperca. Cells, 9(11), 2391. https://doi.org/10.3390/cells9112391
3. *Martorell-Ribera, J., **Venuto, M.T.**, Otten, W., Brunner, R. M., Goldammer, T., Rebl, A., & Gimsa, U. (2020). Time-Dependent Effects of Acute Handling on the Brain Monoamine System of the Salmonid Coregonus maraena. Frontiers in neuroscience, 14. https://doi.org/10.3389/fnins.2020.591738
4. Bornhöfft, K. F. & Martorell-Ribera, J., Viergutz, T., **Venuto, M.T.**, Gimsa, U., Galuska, S.P., & Rebl, A. (2020). Characterization of Sialic Acid-Binding Immunoglobulin-Type Lectins in Fish Reveals Teleost-Specific Structures and Expression Patterns. Cells, 9(4), 836. https://doi.org/10.3390/cells9040836

Contributions to international conferences

1. 28th Joint Glycobiology Conference, Aachen, Germany (09/2017); Poster: "**Characterization of the polysialylation status in fish eggs".**
2. 29th Joint Glycobiology Conference, Ghent, Belgium (10/2018), Poster: **"Towards characterization of the polysialylation status of fish eggs: study of fish α2,8 polysialyltransferases."**
3. GlycoBiotec 2019, Berlin, Germany (01/2019), Poster: "**Towards characterization of the polysialylation status of fish eggs: study of fish α2,8-polysialyltransferases."**
4. XXV International Symposium on Glycoconjugates, Milano, Italy (08/2019), Poster: "**Screening of polysialic acid positive cells during the development of fish larvae and during germ cell maturation in *Coregonus maraena*"**
5. 30th Joint Glycobiology Conference, Lille, France (10/2019), Poster:"**Vertebrate a α,8 sialyltransferase (ST8Sia): a teleost perspective**
6. GlycoT, Boston, Canada (06/2020), Virtual meeting, Poster: '**Characterization of the polysialylation status in fish ovary"**
7. NIH & FDA Glycoscience Research Day 2020, Bethesda, Maryland US, online conference.

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Internal meetings and presentations in FBN

1. Internal FBN institute meeting, (10/2017) Poster: "**Glycans as a modulator of the immune system in the reproductive tract and during pregnancy**"
2. Internal FBN institute meeting, (10/2017) Poster: „**The glycocalyx of fish eggs and sperms: the natural function and a possible source for bioactive natural products**"
3. Internal FBN institute meeting, Day of the Doctoral student, (11/2017) Presentation: "**Characterization of the polysialylation status in fish eggs**."
4. Internal FBN department meeting, (12/2017) Presentation: "**Characterization of the polysialylation status in fish eggs**".
5. Internal FBN department meeting, (12/2018) Presentation, "**Towards characterization of the polysialylation status of fish eggs: structure/function study of fish α2,8-polysialyltransferases**."
6. FBN internal department meeting, (12/2019) Presentation, "**Vertebrate Alfa2,8- Sialyltransferases (ST8Sia): a Teleost perspective**."

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Declaration of honour

"I hereby, declare under that I have completed the work submitted here independently and have I have written it without outside assistance. Furthermore, I have not used anything other than the resources and sources stated and where I have used, the taken sections from these works in terms of content, text or Figure, I have identified this appropriately on the reference."

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Selbständigkeitserklärung

"Hiermit erkläre ich, dass ich die hier vorgelegte Arbeit selbstständig und ohne fremde Hilfe verfasst habe. Darüber hinaus habe ich keine anderen als die angegebenen Quellen und Hilfsmittel benutzt und soweit ich inhaltlich, textlich oder bildlich Teile aus diesen Arbeiten übernommen habe, ich habe dies in der Quellenangabe entsprechend kenntlich gemacht."

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