Brain Distribution of 10 *cart* Transcripts and Their Response to 4 Days of Fasting in Atlantic Salmon (*Salmo salar* L.)

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Cocaine- and amphetamine-regulated transcript (CART) has been known to be involved in feeding and energy balance in mammals, acting as an anorexigenic neuropeptide in hypothalamus. In Atlantic salmon, little is known about Cart brain localization and its function. In this study, *in silico* analysis revealed the existence of 10 *cart* paralogs, here named *cart1a, 1b1, 1b2, 2a, 2b1, 2b2, 3a1, 3a2, 3b,* and *4*. The Atlantic salmon Cart sequences shared from 19 to 50% of identity with the human homolog and between 25 and 90% of sequence identity among paralogs, except for Cart4 which only shared 18–23% of identity. We further explored *cart* mRNA expressions in 8 brain regions (Olfactory Bulb-OB, Telencephalon-TEL, Midbrain-MB, Cerebellum-CE, Hypothalamus-HYP, Saccus vasculosus-SV, Pituitary-PT, and Brain Stem-BS) of Atlantic salmon smolt under 4 days of fasting and continuous fed conditions. The *cart* paralogs analyzed were widely distributed among the brain regions and OB, TEL, HYP, MB, and BS seemed to be the major sites of expression. The expression of *cart1a* and *1b* showed quite similar pattern in MB, HYP, and BS. The expression of *cart2a* had the highest in MB followed by HYP and TEL. The *cart3a* transcript was widely distributed in rostrocaudal regions of brain except in OB and SV whereas *cart3b* was predominantly expressed in BS followed by MB. Expression of *cart4* was high in HYP followed by TEL. With regards to effect of feeding status the Atlantic salmon *cart2b*, which is the most abundant among the paralogs, was upregulated after 4 days of fasting in OB, MB, and HYP compared to fed group. This may suggest an unexpected, but possible orexigenic role of *cart2b* in Atlantic salmon or a fasting induced stress effect. No other significant effect was observed. Collectively, the differential expressions of the *cart* paralogs in different brain regions suggest that they may have roles in regional integration of appetite signals and are possibly involved in regulating other brain functions in Atlantic salmon. The fact that salmon has 10 *cart* paralogs, while mammalians only one, opens interesting perspectives for comparative research on evolutionary adaptations of gene function in the control of appetite and energy homeostasis.

Keywords: appetite, Atlantic salmon, brain, CART, fasting, neuropeptides

INTRODUCTION

The cocaine- and amphetamine-regulated transcript (CART), initially discovered as an unknown peptide isolated from ovine hypothalamus by Spiess et al. (1981), was found to be released in response to the psychomotor stimulants cocaine and amphetamine in rat brain by Douglass et al. (1995) and, thus, its name. The mammalian CART is known to be an anorectic peptide in mediating feeding in accordance with energy balance (Kristensen et al., 1998; Yang et al., 2005; Rogge et al., 2008) but is also involved in stress response, reward and drug addiction/abuse (Koylu et al., 2006).

The CART function and its involvement in appetite control is, however, not so clear for other vertebrates, including teleost fishes. In fish, one cart gene has been previously identified in several species, such as Atlantic cod (*Gadus morhua*) (Le et al., 2016), winter flounder (*Pseudopleuronectes americanus*) (MacDonald and Volkoff, 2009a), winter skate (*Raja ocellata*) (MacDonald and Volkoff, 2009b), Mexican tetra/blind cave fish (*Astyanax mexicanus*) (Penney and Volkoff, 2014), rainbow trout (*Oncorhynchus mykiss*) (Comesaña et al., 2018), channel catfish (*Ictalurus punctatus*) (Kobayashi et al., 2008), silver dollar (*Metynnis argenteus*), bucktooth tetra (*Exodon paradoxus*), black widow tetra (*Gymnocorymbus ternetzi*) (Butt et al., 2019), and Atlantic salmon (*Salmo salar*) (Murashita et al., 2009). In other teleost species, multiple cart transcripts have been identified in the brain including two genes in goldfish (*Carassius auracius*) (Volkoff and Peter, 2001), three in Ya-fish (*Schizothorax prenanti*) (Yuan et al., 2015), four in zebrafish (*Danio rerio*) (Nishio et al., 2012; Akash et al., 2014; Ahi et al., 2019), five in yellowtail (*Seriola quinqueradiata*) (Fukada et al., 2021), six in medaka (*Oryzias latipes*) (Murashita and Kurokawa, 2011), and seven in Senegalese sole (*Solea senegalensis*) (Bonacic et al., 2015).

The existence of multiple cart genes raises questions about their physiological function, and recent studies have suggested that maybe not all the paralogs are involved in the control of feed intake. The cart response to feeding has varied among studies, which can possibly be explained by differences in the method of analysis used, like *in situ* vs. qPCR, or varied methodological approaches like use of whole brain vs. specific brain regions and different development stages, and sampling time related with feeding or fasting. However, it might well be that Cart response to feeding is also species-specific. The differential expression patterns of cart genes in brain regions have been described in some teleost species such as goldfish (Volkoff and Peter, 2001), zebrafish (Nishio et al., 2012; Akash et al., 2014), yellowtail (Fukada et al., 2021), and Senegalese sole (Bonacic et al., 2015). The anorectic effect of cart has been reported for several teleost species, notably in zebrafish where two forms of hypothalamic cart mRNA expressions were downregulated after 5 days of fasting (Akash et al., 2014), and all four identified cart genes expression decreased in the brain after 3 days of fasting (Nishio et al., 2012). Similar cart anorectic effects have been observed in Ya-fish, where the mRNA expression of the three identified cart genes decreased in the hypothalamus after fasting and increased with refeeding (Yuan et al., 2015), while in yellowtail, cart1b and 2a mRNA expression decreased in the telencephalon and

hypothalamus following 8 days of fasting (Fukada et al., 2021). Furthermore, whole brain analysis of cart mRNA expression showed that 6 days of fasting downregulated it's expression in Atlantic salmon (Murashita et al., 2009), while 1 week of fasting decreased cart mRNA in silver dollar but did not affect the expression in bucktooth or black widow tetra (Butt et al., 2019). In medaka, cart2b (previously named cart ch3) expression decreased with 17 days of starvation (Murashita and Kurokawa, 2011).

Atlantic salmon is a key species in Norwegian aquaculture industry and a substantial portion of the production cost, more than 50%, goes to fish feed (Asche and Oglend, 2016). Furthermore, the extensive expansion of the fish industry combined with frequent overfeeding has contributed to potentially negative effects on the ecosystem (Strain and Hargrave, 2005). As such, the efficient utilization of fish feed is vital to ensure sustainable production with regards to the environment, fish welfare, and production costs. Thus, a clear understanding of the biological mechanisms that underly hunger and satiety, and thereby control feed intake, can be of great benefit to the salmon industry. This includes the neuro-endocrine players that modulate feeding and the identification of reliable biomarkers that can be used to assess appetite and potentially growth in fish trials. Moreover, a 2–4 days fasting is implemented prior to aquaculture practices like handling for vaccination, transportation, and harvest (Waagbø et al., 2017). In this regard, learning the impact of fasting will help to optimize the feed utilization, fish growth performance, and welfare. In the current study, we have identified 10 cart paralogs in the Atlantic salmon genome databases and analyzed their brain distribution as well as their response to 4 days of fasting. The results provide a basis for a more comprehensive understanding the role of Cart in the appetite control in Atlantic salmon.

MATERIALS AND METHODS

Ethics Statement
The animal experiment was conducted in accordance with the National Animal Research Authority regulations and approved by the local representative of Animal Welfare at Department of Biological Sciences, University of Bergen, Norway.

Experimental Animals and Sampling
To evaluate the effect of 4 days fasting, we used the experimental design described by Kalananthan et al. (2020b). In brief, Atlantic salmon post smolt (mean body weight 183.81 ± 35.89 g, mean length 25.42 ± 1.68 cm) were divided in two groups, one was kept under continuous feeding (fed group), whereas the other tank was fasted for 4 days (fasted group). Fish were sampled 2 h after feeding for the fed group (n = 6, per tank n = 2) and 4 days of fasting for the fasted group (n = 6, per tank n = 2). Fish were euthanized with a lethal dose of 200 mg/l of MS222 (Tricaine methanesulfonate, Sigma-Aldrich, MO, United States). The whole brain including pituitary was removed from the skull, placed in RNA later (Thermo Fisher Scientific, MA, United States) and stored overnight at 4°C before transferring it to −80°C until further use. Because the key regulatory neural

pathways involving CART in appetite control are found in the hypothalamus of mammals, we have also investigated the hypothalamic *cart* mRNA expression using the total RNA from Kalananthan et al. (2020b) study, which analyzed 3 days fasting vs. normally fed fish groups ($n = 6$ per group).

Sequence Comparison and Phylogenetic Analyses of Cart

Putative Atlantic salmon *cart* genes were retrieved from Ensembl[1] and GenBank[2] genome databases (ICSASG_v2) and compared using ClustalX 2.1 (Larkin et al., 2007).

The Atlantic salmon Cart deduced amino acid (AA) sequences and the homolog sequences from human (*Homo sapiens*) and from 15 other fish species, including the Callorhinchidae, elephant shark (*Callorhinchus milii*); Latimeriidae and Lepisosteidae, spotted gar (*Lepisosteus oculatus*) as species before the teleost specific whole genome duplication event (Ts WGD); two Cyprinidae, goldfish (*Carassius auracius*) as species that went through a very recent 4R WGD and zebrafish (*Danio rerio*) which did not; Characidae, Mexican tetra/ blind cave fish (*Astyanax mexicanus*); Clupeidae, Atlantic herring (*Clupea harengus*) as old teleost; another Salmonidae species, rainbow trout (*Oncorhynchus mykiss*); the Esocidae northern pike (*Esox lucius*) as a sister group of salmonids that diverged before the Ss 4R WGD; six Neoteleostei Gadidae Atlantic cod, Adrianichthyidae medaka (*Oryzias latipes*), Gasterosteidae stickleback (*Gasterosteus aculeatus*), Cichlidae Nile tilapia (*Oreochromis niloticus*) and Scophthalmidae turbot (*Scophthalmus maximus*), Senegalese sole (*Solea senegalensis*), and coelacanth (*Latimeria chalumnae*) were retrieved from Ensembl or GenBank databases.

Cart prepro- and mature peptide sequences were aligned using MUSCLE with the default parameters (UPGMA clustering method, Gap opening penalty −2.90, Gap extension 0.0) from MEGAX (Hall, 2013). The alignments were displayed in GeneDoc 2.7 (Nicholas et al., 1997) and percentages of sequence identity calculated. Putative signal peptides were predicted using PrediSi[3] and proteolytic cleavage sites predicted using Neuropred.[4] Phylogenetic analysis was performed using the predicted mature peptide sequences of Cart. Based on best-fit substitution model analysis in MEGAX, phylogenetic tree was constructed using Maximum Likelihood (ML) with a Jones-Taylor-Thornton (JTT) model (Jones et al., 1992; Kumar et al., 2018) with fixed Gamma distribution (+G) parameter with five rate categories and 500 bootstrap replicates. The tree was rooted to the human CART.

Brain Dissection

Six brains from fed and six from the fasted group of Atlantic salmon were dissected into 8 regions: olfactory bulb (OB), telencephalon (TEL), midbrain (MB), cerebellum (CE), hypothalamus (HYP), saccus vasculosus (SV), pituitary (PT), and medulla oblongata/brain stem (BS) using the same standard dissection protocol as illustrated by Kalananthan et al. (2020a). To ensure high integrity and quality of total RNA, the brain was placed on an ice block during dissection under a zoom stereomicroscope (Olympus SZ51) and cleaned from blood vessels. The brain regions PT, OB, TEL, BS, CE, SV, HYP, and MB were separated in this respective order.

RNA Extraction and cDNA Synthesis

Total RNA was extracted from each dissected brain region using TRI Reagent (Sigma-Aldrich, MO, United States) following the manufacturer's protocol. To avoid any remnants of genomic DNA 5 or 10 µg of total RNA was treated with TURBO DNase-free Kit (Ambion Applied Biosystem, Foster City, CA, United States) using 2 U DNase per reaction. The input amount of total RNA and the reaction volume for DNase treatment was adjusted depending on the available amount of total RNA per region. A NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, MA, United States) and a 2100 Bioanalyzer with RNA 6000 Nano Kit (Agilent Technologies, CA, United States) were used to assess the quantity, purity and integrity of the extracted total RNA. First strand cDNA was synthesized from 1.1 µg of the total RNA using SuperScript III Reverse Transcriptase (Invitrogen, CA, United States) and Oligo $(dT)_{20}$ (50 µM) primers in a total reaction volume of 20 µl for OB, TEL, MB, CE, HYP, PT, and BS ($n = 6$ per group) and for SV ($n = 4$ per group). The hypothalamic total RNA from 3 days fasting and normally fed groups were also performed with DNase treatment and cDNA as described above.

qPCR Set Up and Primer Design

Atlantic salmon *cart* qPCR primers were designed using NCBI primer designing tool (Primer-BLAST[5]) and synthesized by Sigma-Aldrich (St. Louis, MO, United States) (**Table 1**). Wherever possible specific primers were designed spanning an exon-exon junction. Due to the high degree of identity between *cart1b1* and *1b2*; *2b1* and *2b2*; *3a1* and *3a2* transcripts, common primer pairs were designed for these paralogs (**Table 1**). Further, the primers were designed to amplify alternate splice variants (if existing) of the gene. Two genes *actin beta* (*actb*) and *ribosomal protein s20* (*s20*) were used as reference genes (Olsvik et al., 2005). For each primer pair, quantitation cycle (Cq), primer efficiency and melting peaks were analyzed. All qPCR products were resolved in a 2% agarose gel, purified using QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and cloned into a pCR4-TOPO vector (Thermo Fisher, Scientific, Waltham, MA, United States). Sequencing was performed at the University of Bergen Sequencing Facility (Bergen, Norway), and their identity was confirmed using BLASTN[6] analysis against the Atlantic salmon genome database. A standard curve dilution series (10-fold) was generated from each target and reference gene cloned into a pCR4-TOPO vector.

All qPCR assays were performed using 10 µl of iTaq Universal SYBR Green supermix (Bio-Rad, CA, United States), 0.3 µM of each forward and reverse primers, and 2 µl of cDNA template (for

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[1] http://www.ensembl.org/index.html
[2] https://www.ncbi.nlm.nih.gov/
[3] http://www.predisi.de/index.html
[4] http://stagbeetle.animal.uiuc.edu/cgi-bin/neuropred.py

[5] https://www.ncbi.nlm.nih.gov/tools/primer-blast
[6] https://blast.ncbi.nlm.nih.gov/Blast.cgi

TABLE 1 | Sequence of specific primers used for reverse transcriptase quantitative PCR (RT-qPCR) analyses.

Transcripts	Ensembl Gene Acc. No.	GenBank Acc. No.	Chr location	Primer sequences (5′→3′)	Amplicon size (bp)	Efficiency (%)	R^2
cart1a	ENSSSAT00000013202.1	XM_014149393.1	ssa16	F: GGCCAACTCCCACGACTTTC R:CATCAGCATCACACATGGGAACC	170	95.96	0.9997
cart1b	ENSSSAT00000151185.1 ENSSSAT00000086533.1	XM_014150559.1 XM_014151634.1	ssa16 ssa17	F: GGCCAGCATTCAACTGCTTT R: GGAACTCTAGAGCGCGAGTC	104	96.55	0.9997
cart2a	ENSSSAT00000028631.1	N/A	ssa10	F: GGCAAAACTGCAGGGATTGG R: ACATAGGATGGACAGCAGCG	146	93.78	0.9996
cart2b	ENSSSAT00000074136.1 ENSSSAT00000019793.1 ENSSSAT00000019780.1 ENSSSAT00000019807.1	NM_001146680.1 XM_014176449.1 XM_014183838.1 XM_014188599.1	ssa11 ssa26 Unplaced Scaffold	F: TGAGAGACTTCTACCCCAAAGA R: CGTAGGGACTTGGCCGAATT	135	98.78	0.9996
cart3a	ENSSSAT00000060018.1 ENSSSAT00000060001.1 ENSSSAT00000121230.1	XM_014177116.1 NM_001141227.1	ssa27 ssa14	F: GTGATGTTGGAGAGCGGTGC R: CGGGGTCAGTAACAACGCA	112	101.31	0.9997
cart3b	ENSSSAT00000118490.1	XM_014127320.1	ssa11	R: AGAAAGAACTTGTGGGTGCGA R: ACCACAAGGAGGGATCATGC	95	97.58	0.9997
cart4	ENSSSAT00000121235.1	XM_014141614.1	ssa14	F: CGTTCCGTCGTTGGAAACAC R: CCACGTTGGAATTGCACAGA	185	97.49	0.9999
actin beta	ENSSSAG00000001782.1	BG933897.1		F: CCAAAGCCAACAGGGAGAAG R: AGGGACAACACTGCCTGGAT	91	100.61	0.9984
s20	ENSSSAT00000130996.1	BG936672		F: GCAGACCTTATCCGTGGAGCTA R: TGGTGATGCGCAGAGTCTTG	85	98.25	0.9962

Sequence accession number (Ensemble and GenBank databases), chromosome location, primer sequences, amplicon sizes, qPCR efficiency (%), and R^2 are indicated for each primer pair.

target genes 40 ng/reaction and for reference genes 5 ng/reaction) in 20 μl final reaction volume made with ultra-pure water (Biochrom, Berlin, Germany). All the reactions were performed in duplicate, and a non-template control, no-reverse transcriptase control and a positive between plate control were included in every plate. The qPCR was performed using a CFX96 Real-Time System (Bio-Rad Laboratories, CA, United States) in connection to CFX Manager Software version 3.1 (Bio-Rad, Laboratories, CA, United States) with the following conditions: (1) 95°C for 30 s, (2) 95°C for 5 s, (3) 60°C for 25 s, (4) repeating step 2–3 for 39 more times. Melting curve analysis over a range of 65–95°C (increment of 0.5°C for 2 s) allowed for the detection of possible non-specific products and/or primer dimers.

Subsequently, the copy number for each gene/sample were determined based on the respective standard curve and using the following equation:

$$Copy\ number = 10^{\left(\frac{Cq - intercept}{slope}\right)}$$

The copy number was normalized using the total ng of RNA used in the reaction for each target and reference gene. The ratio of the target gene copy number to the geometric mean copy number of reference genes was used in for the plots and statistical analysis.

Statistical Analysis

The data analysis was carried out using GraphPad version 8 (GraphPad Software, San Diego, CA, United States). Normality of distribution and equal variances of gene expression were assessed using Shapiro-Wilk normality test and F-test, respectively. The data were log transformed to ensure normal distribution before
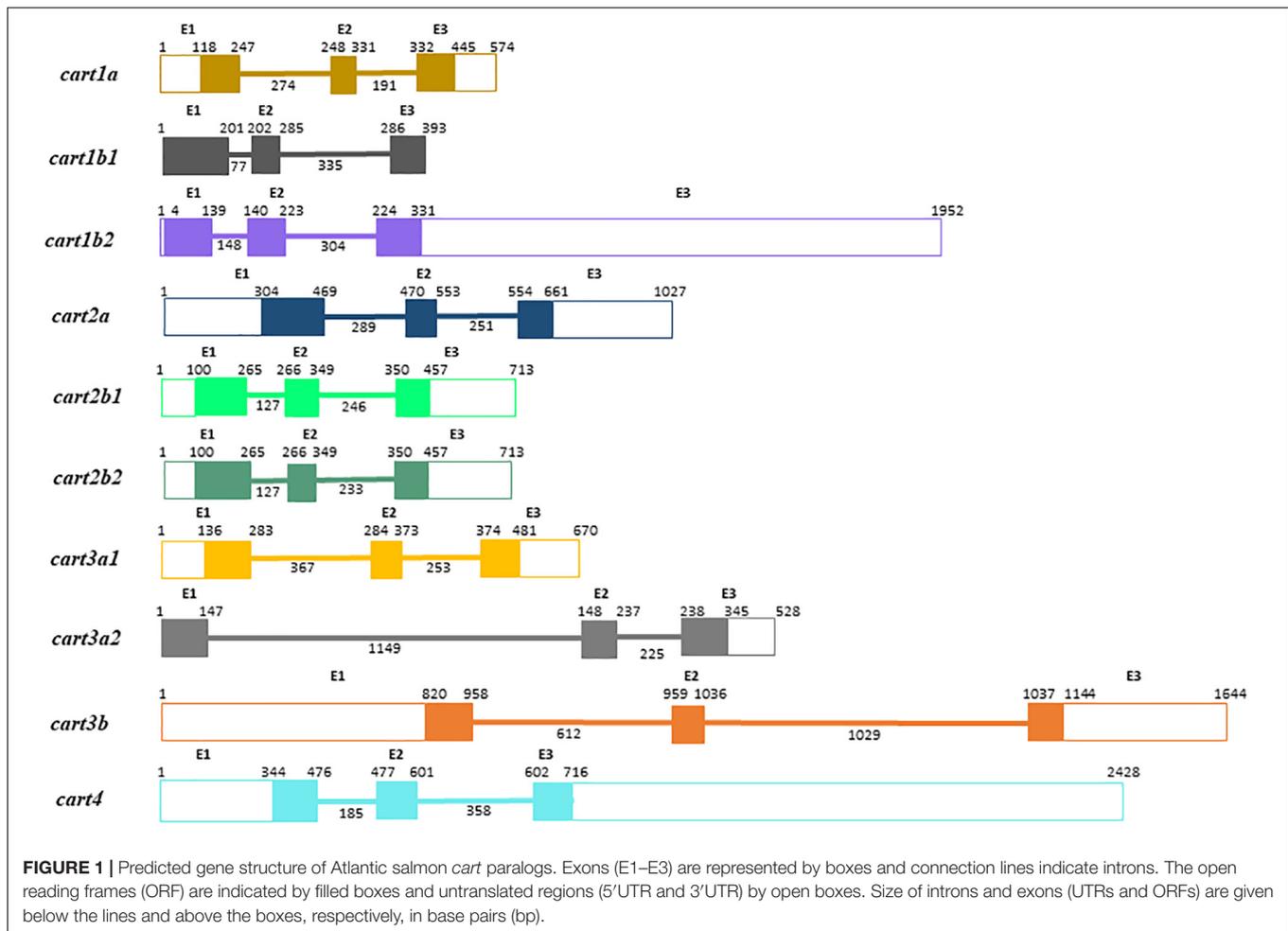
statistical analysis. The analysis of differential expression between the fed and fasted group were performed with two-tail t-test. When either the F-test or the normality test failed, the non-parametric Mann-Whitney test was performed. A $p < 0.05$ was considered significant. All data are presented as mean ± SEM.

RESULTS

Sequences and Phylogenetic Comparison of Cart

In silico searches identified the presence of 10 *cart* paralogous genes in the Atlantic salmon genomic databases. Comparison of the Atlantic salmon *cart* gene structure indicated that gene organization is conserved with three exons encoding the prepro-Cart sequence separated by two introns (**Figure 1**). The coding regions of the identified *cart* genes were 327, 393, 327, 357, 357, 357, 345, 345, 324, and 372 bp (**Figure 1**), translated into 108, 130, 108, 118, 118, 118, 114, 114, 107, and 123 AA, respectively (**Figure 2**). The Cart paralogs were found to be encoded by genes located on chromosomes (Chr) ssa16, ssa17, ssa16, ssa10 (exclusively predicted in Ensembl database), ssa11, ssa26, ssa14, ssa27, ssa11, and ssa14 (**Table 1**). Two alternate splice variants were found for *cart* located in chromosome ssa26, with coding regions of 267 and 510 bp which translate into 88 AA and 169 AA, respectively. One splice variant of *cart* located in chromosome ssa27 was found with a 381 bp coding region that translate into 126 AA.

Phylogenetic analyses revealed that the predicted Atlantic salmon Cart sequences were grouped into 4 major clusters.

FIGURE 1 | Predicted gene structure of Atlantic salmon *cart* paralogs. Exons (E1–E3) are represented by boxes and connection lines indicate introns. The open reading frames (ORF) are indicated by filled boxes and untranslated regions (5'UTR and 3'UTR) by open boxes. Size of introns and exons (UTRs and ORFs) are given below the lines and above the boxes, respectively, in base pairs (bp).

The Atlantic salmon paralogs were named following the nomenclature proposed by Bonacic et al. (2015): Cart1a, 1b1, 1b2, 2a, 2b1, 2b2, 3a1, 3a2, 3b, and 4 (**Figure 3**). As expected, the sequences of rainbow trout (a very close relative to Atlantic salmon) Cart and Northern pike (a basal sister clade to salmonids) Cart cluster very close with Atlantic salmon Cart. The zebrafish and goldfish (cyprinids that are distant relatives to salmonids) Carts formed only 3 major clusters, lacking Cart4. The Atlantic salmon prepro-Cart sequences shared 19–50% of AA with human homolog sequence. Among the Atlantic salmon Cart paralogs, Cart1 sequences shared between 48 and 81% of identity, Cart2 shared 37–77% sequence identity, and Cart3 35–90% AA identity, whereas Cart4 only shared 18–23% of AA sequence identity with the other Cart paralogs (**Supplementary Table 1**).

All the predicted Cart sequences of Atlantic salmon showed a highly divergent N-terminal AA residues with the signal peptide ranging from 19 to 27 AA long, whereas the C-terminal was highly conserved (**Figure 2** and **Supplementary Figure 1**). The human CART proteolytic processing sites of dibasic residues Lys-Arg (KR), Arg-Arg (KK) were conserved in Atlantic salmon Cart2, but only KK site is conserved in Cart1 and Cart3a, while in Cart3b and Cart4, KK is replaced with other proteolytic dibasic residues KR or RR, respectively (**Figure 2**). Furthermore, the 6 cysteine residues that are critical for disulfide bond in human CART are conserved in Atlantic salmon Cart paralogs except for Cart4 where only 5 cysteines are conserved.

Brain Distribution of *cart* mRNA in Atlantic Salmon and Response to Fasting

The qPCR analysis showed that the *cart* paralogs have a wide distribution pattern in the brain with varying abundance between regions (**Figure 4**). The *cart2b* is the most abundant among the *cart* paralogs followed by *cart3b*, *3a*, *2a*, *1b*, *1a,* and *cart4*. For *cart2b* OB, TEL, MB, HYP, SV, and BS were the regions where high levels of mRNA were found, whereas low expression was found in the CE and PT. The *cart3b* was mainly expressed in BS followed by MB. The *cart2a* was prominently expressed in MB, HYP, and TEL whilst *cart4* showed remarkable expression in HYP and TEL. The *cart3a*, *1a,* and *1b* expression profiles were quite similar with major expression in MB, however, *cart3a* was also expressed considerably in BS, TEL, and CE while PT was the second highest site of expression for *cart1a* and *1b*. In the regions OB, TEL, CE and SV for *cart1b*; OB, CE, SV, and PT for *cart2a*; CE and PT for *cart2b*; OB and SV for *cart3a*, and TEL, CE and SV for *cart3b* only residual mRNA expression levels were found.
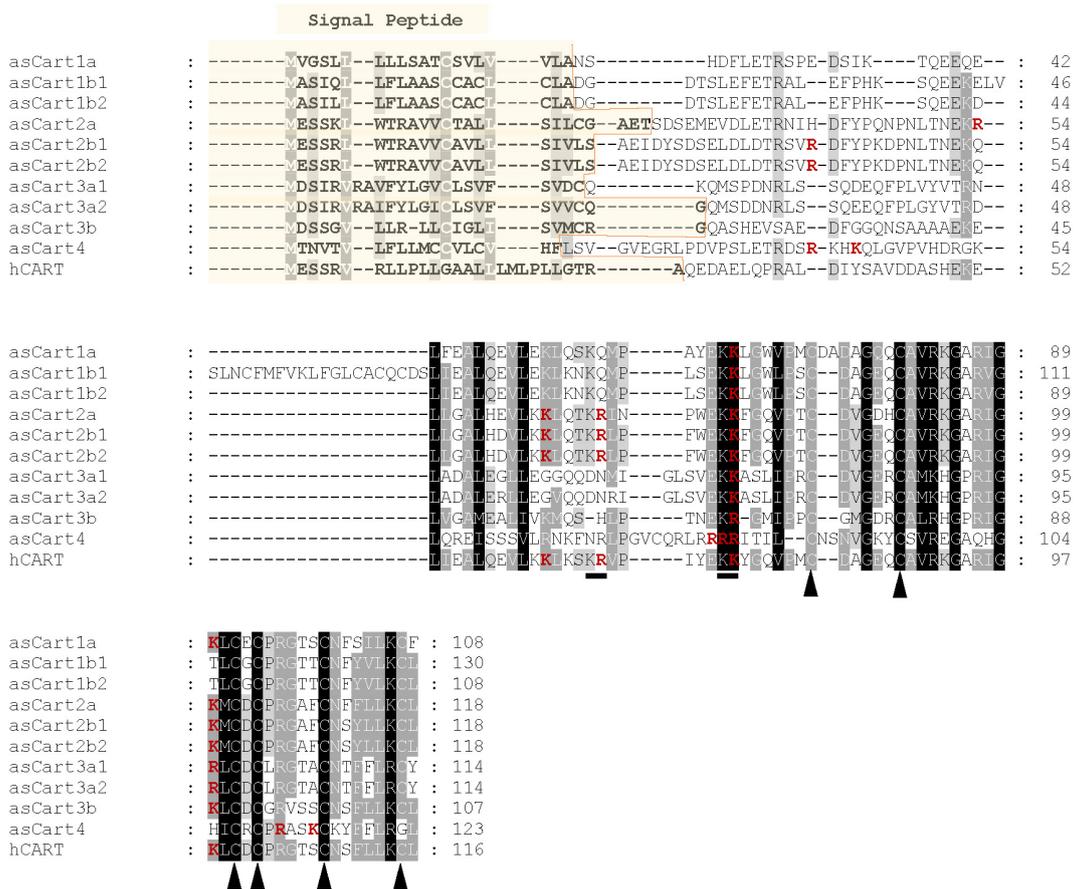
FIGURE 2 | Multiple sequence alignment of Atlantic salmon (as) and human (h) Cart amino acid sequences obtained by using ClustalX 2.1 and edited in GeneDoc 2.7 software. Black areas indicate 100% and shaded areas indicate > 60% conserved amino acid residues among the Cart sequences. The putative signal peptide is highlighted in yellow. Potential proteolytic processing sites KK and KR for hCART are underlined with thick lines and marked in red bold letters are the predicted sites for Cart using Neuropred application. Arrowheads indicate the conserved 6 cysteine residues that forms the disulfide bond in human CART.

Atlantic salmon *cart2b,* which is the most abundant *cart* paralog, was upregulated in OB ($p < 0.05$), MB ($p < 0.01$), and HYP ($p < 0.05$) after 4 days of fasting (**Figure 4**). No effect of fasting was observed for the other *cart* paralogs in any of the highly expressed brain regions (**Supplementary Table 2**).

Among the *cart* paralogs that were tested for the effect of 3 days of fasting in hypothalamus of Atlantic salmon, *cart3a* showed significant ($p < 0.01$) increase in the mRNA expression (**Figure 5**).

DISCUSSION

In this study, we report the identification and characterization of 10 *cart* paralogs in Atlantic salmon, here named *cart1a, 1b1, 1b2, 2a, 2b1, 2b2, 3a1, 3a2, 3b,* and *cart4*. This is the first study to analyze all the aforementioned paralogs physiological role in Atlantic salmon appetite control, while the previously reported Atlantic salmon Cart by Murashita et al. (2009) is here denoted as Cart2b (b1 and b2). Furthermore, the mRNA sequences of the qPCR amplicons confirmed that none of salmon *cart* genes

are pseudogenes. The deduced amino acid sequences showed a highly divergent N-terminal AA residues whereas the C-terminal showed a high degree of conservation. Among Cart paralogs, the potential proteolytic processing sites of dibasic residues KR and KK (Kuhar and Yoho, 1999; Rogge et al., 2008) are conserved only in Cart2, possibly cleaving it into two different size products as previously suggested by Murashita et al. (2009). In previous studies, Kuhar and Yoho (1999) identified four immunoreactive CART peptides from human and five from rat hypothalamus varying from 4 to 10 kDa. The predicted cleavage sites for Atlantic salmon Cart (**Figure 2**) also suggest possible multiple Cart peptides. The 3 cysteine disulfide bonds between Cys[82] and Cys[100], Cys[88] and Cys[108], Cys[102], and Cys[115] were predicted for human CART C-termini (UNIPROT[7]). These six C-terminal cysteines are conserved in all the Atlantic salmon Cart, except Cart4 where only 5 cysteine residues are present. The absence of C- terminal cysteine bond among the homologs has not been reported in teleosts before. The three disulfide bridges are key residual structures that are essential for the protein 3-dimensional
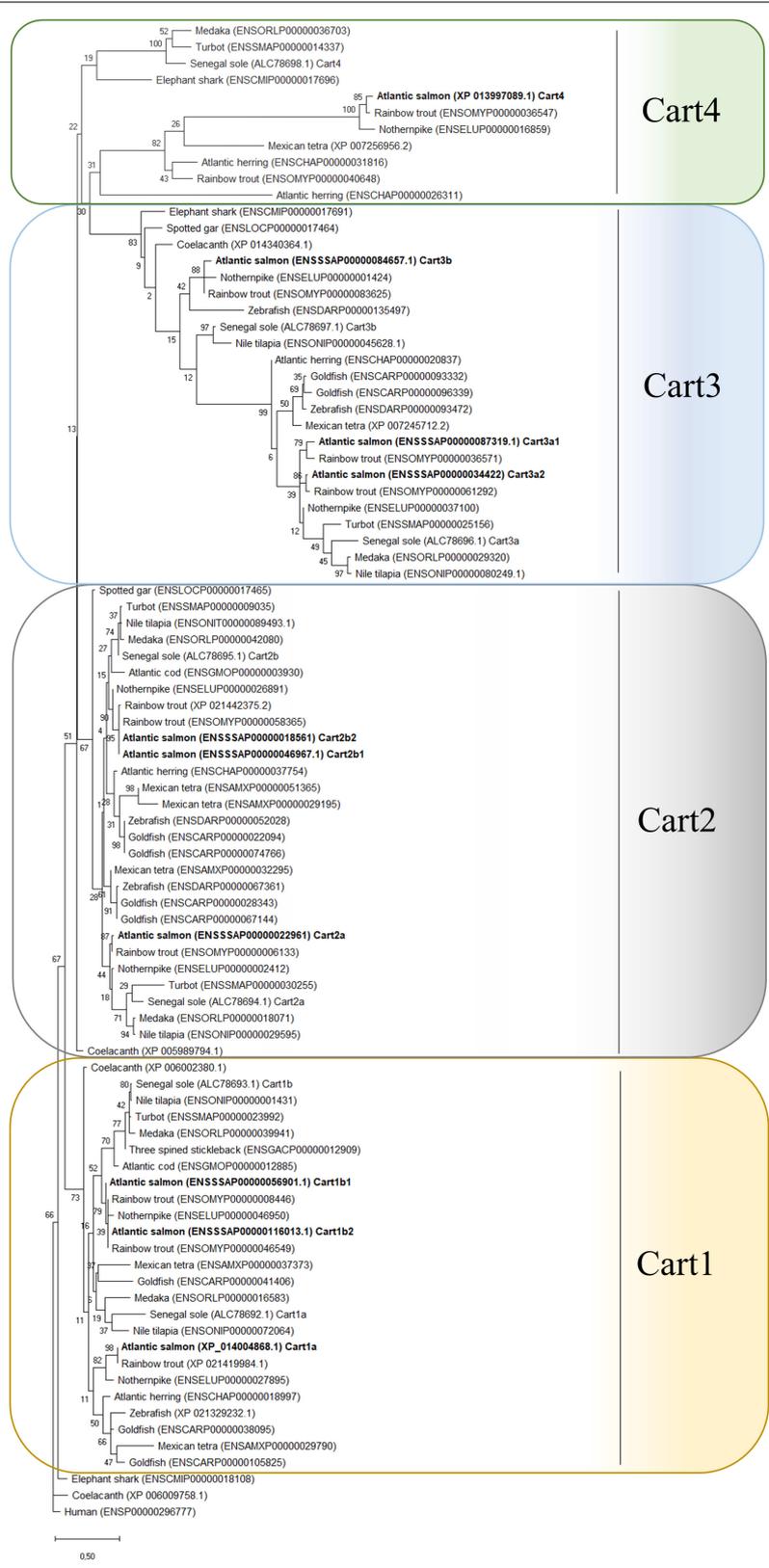
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[7]https://www.uniprot.org/uniprot/Q16568

FIGURE 3 | Phylogenetic analyses of Atlantic salmon Cart and other fish species and human based on the predicted mature peptide sequences. The evolutionary history was constructed by using the Maximum Likelihood method, 500 bootstraps replicates and JTT + G matrix-based model in MEGA X. The tree with the highest log likelihood (–4018, 51) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 91 amino acid sequences. The tree is rooted to human CART.
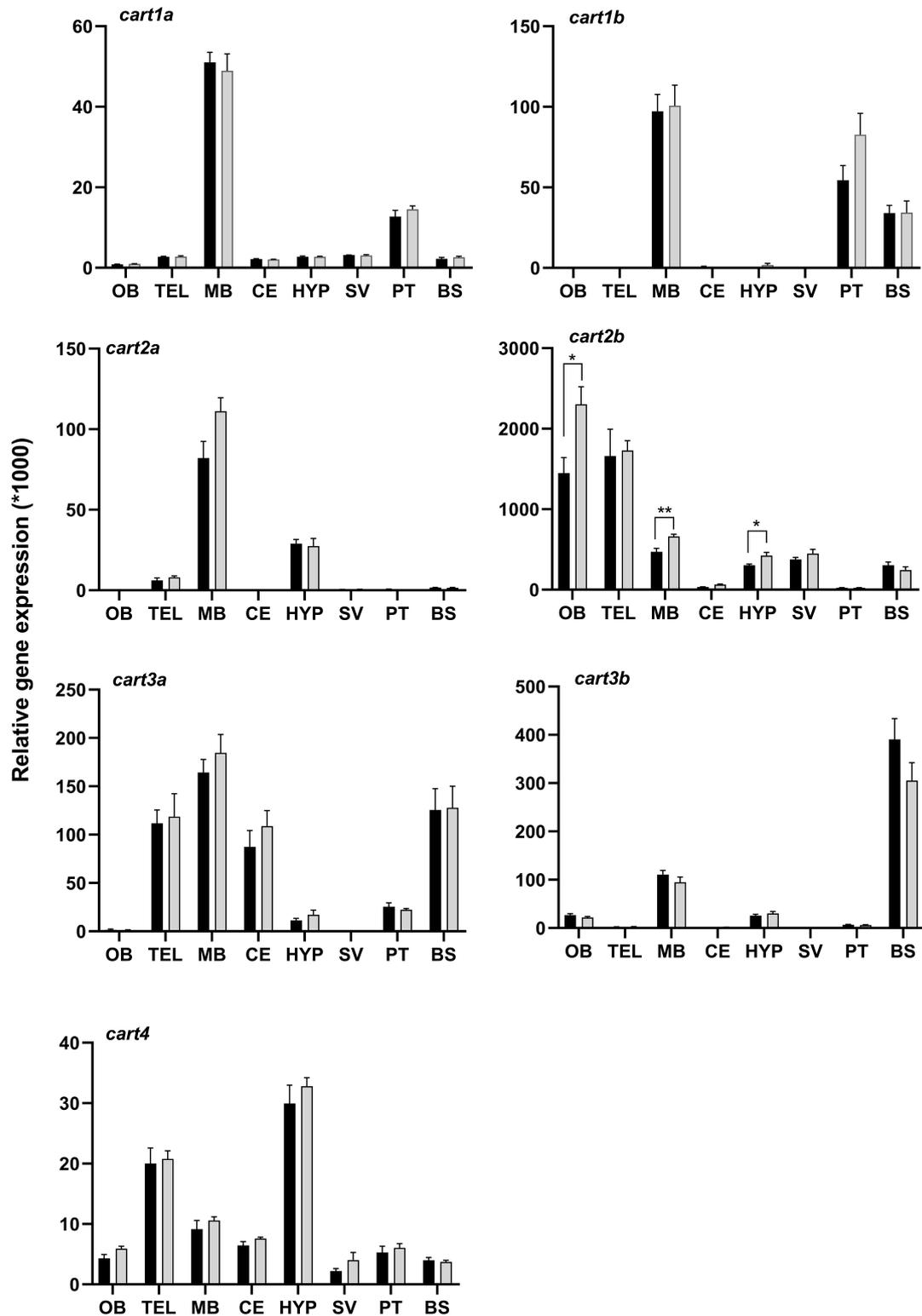
FIGURE 4 | Effect of 4 days of fasting on the mRNA expression of *cart* paralogs in eight brain regions of Atlantic salmon. Black and gray bars represent fed ($n = 6$ except for SV $n = 4$) and fasted ($n = 6$ except for SV $n = 4$) groups, respectively. The graphs show the normalized *cart* mRNA copy number to the geometric mean copy number of *actin beta* and *s20*. Two-tailed *t*-test and non-parametric Mann-Whitney test was performed to assess the statistically significant differences between the two groups. The error bars represent mean ± SEM and *asterisks* show the significant level (*$p < 0.05$, **$p < 0.01$). OB, olfactory bulb; TEL, telencephalon; MB, midbrain; HYP, hypothalamus; CE, cerebellum; SV, saccus vasculosus; PT, pituitary; BS, brain stem.
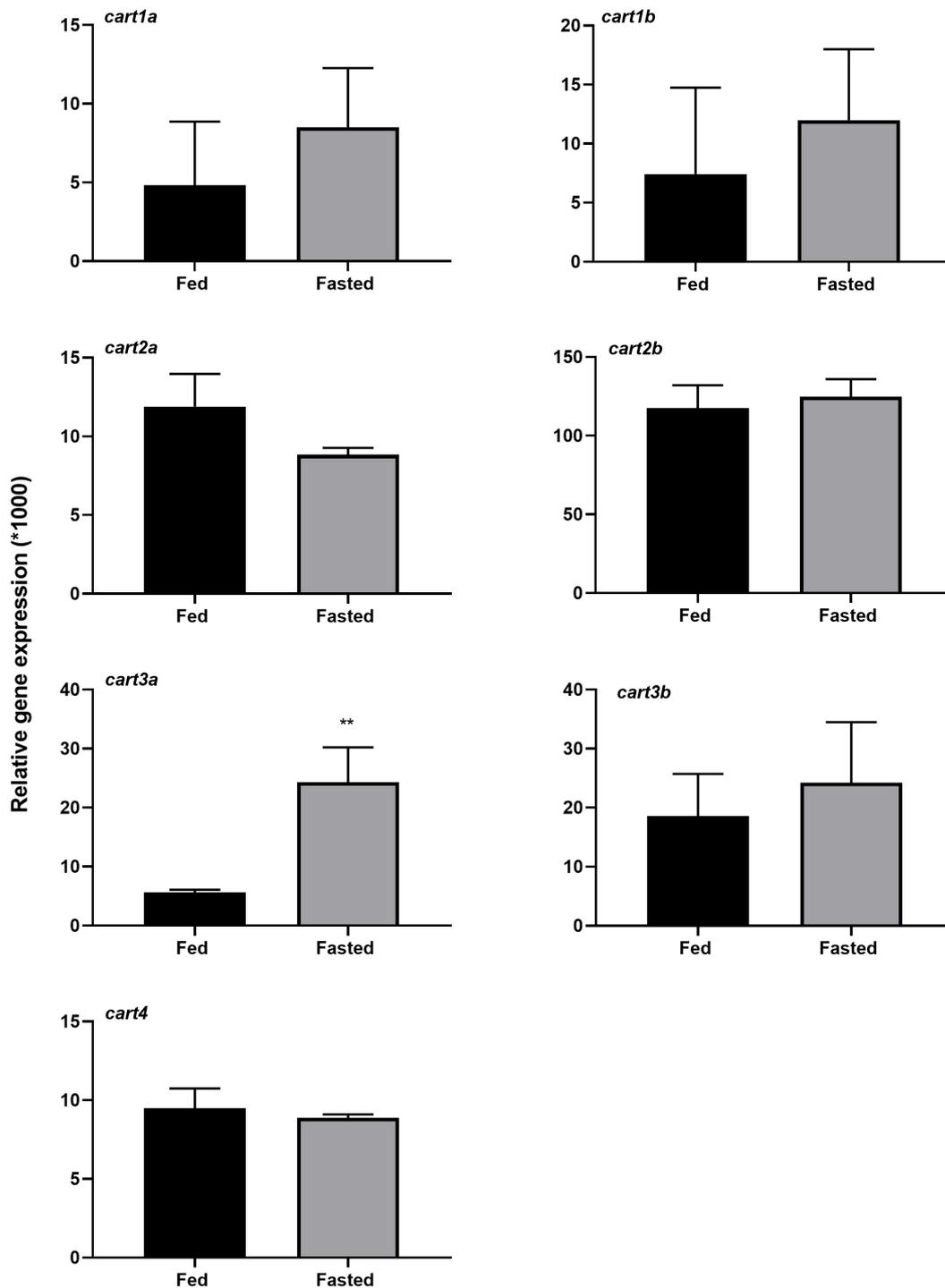
FIGURE 5 | Effect of 3 days of fasting on mRNA expression of *cart* paralogs in hypothalamus of Atlantic salmon. Black and gray bars represent fed (*n* = 5–6) and fasted (*n* = 5–6) groups, respectively. The graphs show the normalized *cart* mRNA copy number to the geometric mean copy number of *actin β* and *s20*. Two-tailed *t*-test and non-parametric Mann-Whitney test was performed to assess the statistically significant differences between the two groups. The error bars represent mean ± SEM and *asterisks* show the significant level (**$p < 0.01$).

structure and its function in biological activities such as food intake (Ludvigsen et al., 2001; Maixnerová et al., 2007). The Atlantic salmon Cart sequence alignment clearly show that Cart4

structure potentially differs from the other salmon's Carts and human CART. This suggests that Atlantic salmon Cart4 may differ in functionality from the other Carts and may likely be

ruled out from its role in appetite regulation. It would also be possible that Cart4 is non-functional or has adopted new function and this warrants further studies. In teleost *cart* mRNA expression in peripheral tissues like eye, gill, ovary, testis has also been reported suggesting Cart may also have other roles (Volkoff and Peter, 2001; Murashita et al., 2009). Interestingly, the multiple sequence alignment of Cart prepropeptides revealed also Cart4 in northern pike, rainbow trout, Atlantic herring and Cart2 in Atlantic cod lack of one or more Cysteine residue in the C-termini (**Supplementary Figure 1**).

The evolutionary history of *cart* depicted in the phylogenetic tree shows that the Ts 3R genome duplication resulted in the presence of 7 *cart* genes in species like Senegalese sole, Mexican tetra, Northern pike, etc., and the Ss 4R genome duplication resulted in 10 *cart* genes in the Atlantic salmon, including sub types *cart1b1* and *1b2*, *cart2b1* and *2b2*, *cart3a1* and *3a2*. The divergence of the *cart* gene in salmonids opens the possibility that the gene products might have adopted to new functions (neofunctionalization) or partitioning the ancestral function among the paralogs (sub functionalization) during genome evolution reviewed by Volff (2005). Furthermore, the fact that Atlantic salmon and other teleosts have multiple *cart* genes while only one is present in mammals', open up interesting perspectives for comparative research on evolutionary adaptations of gene function and the control of appetite and energy homeostasis.

To date, there are only a few studies exploring the full *cart* gene repertoire in teleost species. These studies include the identification of seven *cart* paralogous genes in Senegalese sole (Bonacic et al., 2015), six genes in medaka (Murashita and Kurokawa, 2011), and five yellowtail (Fukada et al., 2021). Thus, a revision of the teleost genome databases and nomenclature of *cart* genes is essential in further studies exploring the functional role of Cart in these species.

Our results revealed that all *cart* paralogs were expressed at different mRNA levels in the eight brain regions analyzed in this study. Among all the *cart* genes, *cart2b* was the most abundant paralog, with predominant expression in the OB followed by TEL, MB, HYP, and SV. A similar pattern has also been reported in Senegalese sole (Bonacic et al., 2015) and yellowtail (Fukada et al., 2021). In teleosts, Cart has been suggested to play a role in processing olfactory information (Akash et al., 2014; Porter et al., 2017) including antiviral immune response (Sepahi et al., 2019). However, the Atlantic salmon *cart2b* expression in the OB and a possible link to olfaction and/or immune response is not clear and needs to be further investigated. The c*art2b* was the only gene that was affected by 4 days of fasting, and a significantly higher expression was observed in the fasted group compared to the fed group in the Atlantic salmon specifically in OB, MB, and HYP. Hypothalamus is considered as a center of appetite control in vertebrates (Volkoff, 2016; Rønnestad et al., 2017; Soengas et al., 2018), and in fish this is supported by Ye et al. (2020) who reported that in grass carp the hypothalamus and telencephalon might be involved in feeding and reproduction. Hence, in Atlantic salmon, hypothalamic *cart2b* might play a vital role in appetite regulation, with fasting maybe inducing shutting down hunger. This is in line with observations in Siberian sturgeon (*Acipenser baerii*) (Zhang et al., 2018), where

cart mRNA in whole brain increased after 3–17 days of food deprivation. Interestingly, our finding is contrary to several previous studies that demonstrate the anorectic function of Cart. In goldfish, *cart* form I (*alias cart2b*) mRNA level declined with 96 h food deprivation in telencephalon, hypothalamus, and olfactory bulb while *cart* form II (*alias cart2a*) mRNA level declined only in the olfactory bulb. In the same study, *cart2b* transcript levels increased 2 h following a meal in olfactory bulb and hypothalamus whereas no postprandial changes were observed in *cart2a* (Volkoff and Peter, 2001; Volkoff et al., 2005). However, in yellowtail *cart2b* expression in OB, TEL, HYP was upregulated in fish fasted for 8 days, though not significantly, while *cart3a* was significantly upregulated in OB, TEL, and PT of the fasted group (Fukada et al., 2021). The brain *cart* mRNA expression in response to fasting varied depending on species, and even within the same order as for example, in silver dollar, fasting induced a decrease in *cart* expression while for black widow tetra and bucktooth no effect was observed (Butt et al., 2019). Forebrain *cart* mRNA expression levels were significantly higher in cod fish held at 2°C than in those held at either 11 or 15°C (Kehoe and Volkoff, 2008) suggesting Cart mediates temperature- induced changes in appetite during the seasonal changes in the lifespan of this species. In addition, the stomach from fish acclimated to 2°C had less food. Taking into consideration that these fish were handfed to satiety, the results suggested Cart acts as a satiety factor in this species (Kehoe and Volkoff, 2008). Previous studies investigating the role of *cart* in appetite regulation in Atlantic cod (Kehoe and Volkoff, 2007, 2008) and Atlantic salmon (Murashita et al., 2009) using a single *cart* paralog suggested an anorectic role for the neuropeptide. Indeed, in Atlantic salmon, 6 days of fasting resulted in decrease of brain *cart2b* (previously named *cart*) (Murashita et al., 2009). In medaka, 17 days of fasting also downregulated brain *cart2b* (previously named *cart ch3*) (Murashita and Kurokawa, 2011). However, both studies have used whole brain in the gene expression analysis. Accordingly, *cart2b* seemed to be involved in appetite regulation but the available data on its action are contradictory since orexigenic, anorexigenic or no action have been reported depending on whether the analyses were performed at brain region level (for e.g., hypothalamus) or on the whole brain and/or the fasting time used. Besides, two different studies in zebrafish reported that *cart2a* and *cart3a* (previously named *cart2* and *cart4*, respectively, Akash et al., 2014) were downregulated in response to short-term fasting within hypothalamic region and *cart2b* (previously named *cart3*, Ahi et al., 2019) was downregulated in brain after 7 days of fasting. The involvement of Cart2 in appetite regulation in Atlantic salmon is further supported by the fact that this is the only Cart paralog that has the same potential proteolytic sites as in mammals. These studies show the diversity between species and importance of long-term vs. short-term testing of the Cart neuropeptide and emphasizes the need for more sampling time points to uncover the specific role of Cart paralogs in appetite regulation in Atlantic salmon.

The *cart1a* and *1b* show quite similar mRNA expression profile and *cart1*, *cart2a*, and *cart3a* are highly expressed specially in the MB, suggesting that these *cart* paralogs might share the

same functional role. Comparably, in Senegalese sole, Bonacic et al. (2015) observed that *cart1*, *cart2a,* and *cart3a* were highly expressed in mesencephalon which is the equivalent region to the MB in our study. The *cart1a* and *1b* are primarily expressed in MB followed by PT in Atlantic salmon brain, which indicate that these genes might be involved in functions linked to these brain regions, such as vision and feeding for MB, and energy metabolism, organ development and reproduction for PT. Ye et al. (2020) showed that in grass carp the optic tectum seems to play an important role in the visual system and feeding. The potential role of *cart* in reproduction is reported in chicken, where pituitary CART peptide expression and secretion is regulated in the hypothalamic-pituitary-gonadal (HPG) axis through gonadotropin-releasing hormone (GnRH), and GnRH administration increased plasma CART (Mo et al., 2019). In this study, the *cart2a*, *2b,* and *4* mRNA expression in hypothalamus suggests that these might be involved in appetite control in Atlantic salmon. We further speculate that they might relay the signals relevant to feeding at different time points. However, further studies are needed to unravel the actual molecular interaction and functionalization among *cart* paralogs.

The feeding regime (continuously or meal-based) and the period in between last feed and sampling are also reflected in the expression of neuropeptides regulating appetite in the brain. Most probably, there is a higher variation in voluntary feed intake when the fish are fed continuously. The macronutrient composition of the feed and the period of fasting are also important factors as it can influence the species digestive physiology and gut evacuation time, and consequently affect the appetite control. Fukada et al. (2021) reported that in yellowtail, *cart2a* showed a tendency to decrease in hypothalamus with fasting and increased with refeeding when the sampling was performed 3 h after feeding. Similarly, in our study, 3 days of fasting resulted a tendency to downregulation of *cart2a* in HYP in fasted group compared to the fed group (sampled 2 h after last feed).

The *cart3b* expression revealed a completely different profile compared to other paralogs with BS as its primary site of expression of this paralog in the brain. This is in line with particularly high expression levels detected in the spinal cord in Senegalese sole (Bonacic et al., 2015). Though there was no significant difference in *cart3b* mRNA expression between the fed and fasted group, the decreasing trend with fasting in BS shows a potential anorexigenic role. We speculate that satiety information generated during a meal, largely conveyed via afferent fibers of vagus nerves first to the hindbrain from the upper gastrointestinal tract in mammals (reviewed in Schwartz et al., 2000), seems to be conserved in fish species. The BS region which contains Mauthner neurons (MN), specific multifunctional neurons in fish, receives afferent information from vestibular, auditory, and visual analyzers and can integrate the afferent activity. In Chinese sleeper (*Perccottus glehni*), the MN responded to severe environmental conditions like deficit of nutrients/energy substrates, oxygen and temperature changes by modifying the structure of the cell and through different pathways for the maintenance of the energy balance (Santalova et al., 2018).

Whether this link between *cart3b* and MN is also seen in Atlantic salmon brain under starvation is unknown and would be an interesting topic for further studies.

Cart has also been suggested to be involved in other physiological processes like stress response, anxiety, depression, reproduction (Koylu et al., 2006), and immune response (Burgos et al., 2019) in mammals.

The interlink between CART peptide and other stress related peptides in the hypothalamic-pituitary-adrenal (HPA) axis have been somewhat studied in mammals and the findings show that intracerebroventricular CART injection increases hypothalamic *corticotropin-releasing factor* (*crf*) mRNA levels and CRF release. In rats, centrally administered CART increased adrenocorticotropic hormone and corticosterone in circulation and exerted dose-dependent increase in anxiety-related behaviors (reviewed in Koylu et al., 2006). Similarly, in fish under stressed conditions the mechanisms involving food intake are deregulated. Consequently, the regular signals controlling appetite in the brain are altered, resulting in changes in the expression of the appetite-related neuropeptides and reduction in food intake (reviewed in Conde-Sieira et al., 2018). In zebrafish, exposure to acute handling stress induced increased expression of *cart* and *pro-opiomelanocortin (pomc)* as central keys in the appetite control (Cortés et al., 2018), but the response of appetite regulating neuropeptides to stress can differ in salmonids (reviewed in Conde-Sieira et al., 2018). In our study, both experimental groups of fish were maintained throughout the experiment under same rearing conditions, even during sampling both groups went through the same procedures. The effect of handling stress in the appetite regulating neuropeptides genes expression in Atlantic salmon brain still remains to be uncovered.

CONCLUSION

This study identifies 10 *cart* paralogs in Atlantic salmon brain with distinct distribution profile. Among the paralogs *cart2b* responded to 4 days of fasting by increase in mRNA expression in hypothalamus, olfactory bulb and midbrain while *cart3b* showed a trend of decline in brain stem. The difference in key residual structure of Cart4 suggesting potentially different function other than appetite regulation. The Cart paralogs 1a, 1b, 2a, and 3a might likely have potential different roles in the regional integration of appetite (relaying) signals in the hypothalamus, telencephalon, and brain stem, and/or other functions in the olfactory bulb and midbrain. To uncover their specific role in appetite regulation in Atlantic salmon more sampling time points will be required. Moreover, the complex molecular interactions with other peptide hormones need to be untangled to understand the potential role of Cart in appetite regulation in Atlantic salmon.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in **Supplementary Table 3**.

ETHICS STATEMENT

AUTHOR CONTRIBUTIONS

TK, AG, KM, IT, FL, and IR conceived and designed the study. AG, KM, and IR conducted the experiment. AG, TK, KM, and FL contributed to the sampling. TK and AG did the primers design and phylogenetic analysis. TK and IT did preparatory lab work and qPCR. TK performed statistical analysis and drafted the manuscript. All authors contributed to the interpretation of the data, writing of the manuscript, read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2021.763766/full#supplementary-material

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