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Molecular characterization and gene expression of synaptosome-associated protein-25 (SNAP-25) in the brain during both seaward and homeward migrations of chum salmon *Oncorhynchus keta*

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ABSTRACT

It is generally accepted that information about some of the odorants in the natal streams of anadromous Pacific salmon (Genus *Oncorhynchus*) is imprinted during their seaward migration, and that anadromous Pacific salmon use olfaction to identify their natal streams during the homeward migration. However, little is known about the molecular mechanisms of the various pre-synaptic functions that are important for olfactory imprinting and memory retrieval in the salmon brain. Synaptosome-associated protein-25 (SNAP-25) mediates pre-synaptic vesicle exocytosis and regulates synaptic transmission and neuronal plasticity. Despite the importance of synaptic plasticity for memorization, the expression of SNAP-25 in the salmon brain is not well understood. In this study, *snap25* expression was detected in chum salmon (*O. keta*) brains using molecular biological techniques. Two cDNAs encoding salmon SNAP-25 were isolated and sequenced (SNAP-25a and SNAP-25b). These cDNAs encoded proteins with 204 amino acid residues, which showed marked homology with each other (97%). The protein and nucleotide sequences demonstrated a high level of homology between salmon SNAP-25s and those of other teleost species. By quantitative PCR, the expression of *snap25a* and *snap25b* was detected in all regions of the salmon brain, especially in the telencephalon. The expression levels of *snap25a* in the olfactory blub were higher during seaward migration than in upriver and post-upriver migrations, reflecting synaptogenesis in the olfactory nervous system, and *snap25b* in the telencephalon was increased during upriver period. Our results indicated that *snap25s* gene is involved in synaptic plasticity for olfactory imprinting and/or olfactory memory retrieval in Pacific salmon.

Key words: Neurotransmission - Olfactory imprinting - Salmon - Migration - Pre-synaptic exocytosis - SNAP-25 - Teleostei

1. Introduction

Homing migration of anadromous Pacific salmon (Genus *Oncorhynchus*) is an especially fascinating phenomenon in fish biology and also of importance for fisheries and game fishing. In the final phase of homing migration, mature adult salmon discriminate their natal river based on olfaction in the coastal area and then continue homing. The widely accepted "olfactory imprinting hypotheses" (Wisby and Hasler, 1954) indicates that juveniles imprint the odor of their natal stream during their seaward migration, and then the homing adult uses olfaction to discriminate their natal river (Hasler and Scholz, 1983; Dittman and Quinn, 1996). Olfaction-dependent homing is conducted rigorously, and mature adult can locate their natal habitat with high precision (Quinn, 2005). As chemical cues to their natal river, it has been suggested that river-specific odorants and their composition are involved (Ueda, 2011), and dissolved amino acids in river water were the first chemicals shown to function as natal river odorants (Shoji et al., 2000, 2003; Yamamoto et al., 2013). These external olfactory cues are received by olfactory receptors expressed in olfactory sensory neurons (Satou, 1990), which proliferate dramatically during the seaward migration stage in chum salmon (*O. keta*) (Kudo et al., 2009). The salmon olfactory nervous system is composed of peripheral (olfactory organ) and central (olfactory center; olfactory bulb, and telencephalon including higher center) regions, which are similar to other teleosts (Satou, 1990). The central nervous system in the seaward migration stage exhibits high neuronal plasticity through alterations in the external and internal environments, which regulate olfactory imprinting (e.g., Dittman et al., 1996; Nevitt and Dittman, 1998; Ebbesson and Braithwaite, 2012; Ueda et al., 2016). The olfactory center is involved in olfactory imprinting in the natal river in the seaward-juvenile stage (Yamamoto et al., 2010, 2013), and with olfactory memory retrieval of the natal river odorants in spawning adults (Bandoh et al., 2011). Recently, transcriptome analyses of the olfactory organ revealed the expression of olfactory-related genes during the seaward and homeward migrations in chum salmon (Palstra et al., 2015). In order to understand the mechanism of olfactory imprinting, neuronal molecular biological studies of salmon olfactory center should be effective. However, little is known about the functional molecules associated with neurotransmission that are important for olfactory imprinting and memory retrieval in the salmon brain. There are a few known examples, such as N-methyl-D-aspartic acid (NMDA)-type glutamate receptor NR1 subunit mRNA expression in chum salmon brain increased during seaward (Yu et al., 2014) and upriver migration (Kim et al., 2015). Generally, the NMDA receptor and glutamatergic synapses are associated with long-term potentiation of synapses (e.g., Siegelbaum and Kandel, 1991), thus increasing *nr1* expression levels in the juvenile brain, which suggested an association with long-term potentiation and subsequent imprinting (Kim et al., 2015; Ueda et al., 2016). Furthermore, brain neurotransmitter levels

were increased in the brain during the parr–smolt transformation of young coho salmon (*O. kisutch*) (Ebbesson et al., 1992, 1996). However, pre-synaptic molecules and these functions were not studied in salmonid brains, although neurotransmitter exocytosis occurs prior to its reception.

Exocytosis of pre-synaptic neurotransmitter vesicles is regulated by the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex (Lin and Scheller, 2000). Synaptosome-associated protein-25 (SNAP-25) is a component of the SNARE complex, which regulates synaptic transmission and neuronal plasticity, and is associated with learning or memorization in rat (*Rattus norvegicus*) and mouse (*Mus musculus*) (e.g., Osen-Sand et al., 1993; Johansson et al., 2008; Zhang et al., 2014). It was reported that SNAP-25 mRNA and protein expression levels corresponded with the time of synaptogenesis in chicken (*Gallus gallus domesticus*) retinas (Catsicas et al., 1991). SNAP-25 accumulates in mouse brain insular and somatosensory cortices during reactions to food and chemical stimulation (Kawakami et al., 2012). Repeated formaldehyde inhalation decreased SNAP-25a and -25b protein expression levels in the mouse olfactory bulb and also impaired learning abilities to detect buried food (Zhang et al., 2014). Therefore, SNAP-25 may be involved in the reception of chemical stimulation, including olfactory stimulation in the salmon olfactory nervous system. Vertebrate *snap25* has a tandem duplication of exon 5, and its alternative splicing introduced two isoforms, *snap25a* and *snap25b* (Bark, 1993; Bark and Wilson, 1994; Risinger et al., 1993b, 1998). Exon 5 of *snap25* is characterized by four cysteine residues involved in post-transcriptional modification, which is essential for its membrane localization and function (Washbourne et al., 2001) or membrane docking (Prescott and Chamberlain, 2011). SNAP-25s (or *snap25s* genes) were also reported to have differential protein and mRNA expression patterns in the central nervous system in a spatiotemporal manner (e.g., Bark et al., 1995; Boschert et al., 1996). In higher vertebrate brains, SNAP-25a protein and mRNA expression was distributed in rostral regions (i.e., olfactory bulb, telencephalon, and thalamus), whereas SNAP-25b expression was high in the caudal region (i.e., optic tectum, cerebellum, and medulla) (Boschert et al., 1996; Prescott and Chamberlain, 2011; Yamamori et al., 2011). Fetal development in rodents involves brain *snap25s* expression with a temporal increase in *snap25a* levels and post-natal *snap25b* expression as the major isoforms in the maturing central nervous system (Johansson et al., 2008; Prescott and Chamberlain, 2011). SNAP-25s are associated with neuroplasticity and memorization, and may have isoform-specific functions (Delgado-Martínez et al., 2007; Yamamori et al., 2011), furthermore, SNAP-25a localization in the olfactory bulb suggests that SNAP-25a is a major SNAP-25 isoforms in olfaction. In teleosts, some previous studies have isolated *snap25* nucleotide sequences (e.g., zebrafish *Danio rerio*: Rinsinger et al., 1998).

The conserved structures of teleost SNAP-25 proteins are also discussed based on multiple bioinformatics analysis methods rather than X-ray crystallography (Chen and Huang, 2007). The sequence of salmon SNAP-25 had been determined through genomic analysis of Atlantic salmon (*Salmo salar*) (Leong et al., 2010). Despite the importance of SNAP-25s in neurotransmission and olfactory imprinting, the expression of *snap25* is not well understood in the salmon brain.

Our objective was to reveal the relationship between *snap25s* expression and olfactory imprinting/memory retrieval in anadromous Pacific salmon. In this study, we identified chum salmon *snap25* nucleotide sequences and analyzed expression changes during their imprinting and/or memory retrieval periods for the natal stream using molecular biological techniques.

2. Materials and methods

2.1. Animals

snap25s expression levels in the chum salmon brain and olfactory organs were examined during 7 different migration periods in its life history: 1) pre-seaward, 2) seaward, 3) post-seaward, 4) feeding, 5) pre-upriver, 6) upriver, and 7) post-upriver. Pre-seaward and seaward juveniles were collected from the upper (42°17'29"N, 140°07'43"E) and lower (42°15'31"N, 140°15'16"E) reaches of the Yurappu River in March and April 2016 and 2017 using an electric fisher (LR-24 Electrofisher, Smith Root). Post-seaward juveniles were reared in artificial seawater (Tetra) in a 60 L glass tank with aeration and filtration in a constant temperature room at 10°C (Faculty of Fisheries Sciences, Hokkaido University) for 2 weeks from above seaward juveniles in 2016 and 2017. Feeding immature fish were collected from the Northwest Pacific Ocean (43°01'N, 155°00'E) by the training ship "Oshoryo-Maru V" (Hokkaido University) in May 2017. Pre-upriver maturing fish were captured using set nets in coastal waters off Yakumo Town (42°15'33"N, 140°17'17"E), southwestern Hokkaido, in October 2016. Upriver and post-upriver mature fish were collected from the lower and upper reaches of the Yurappu River using the electro fisher in November 2016, respectively. Body size (fork length; distance from the tip of the snout to the center of the fork in the caudal fin) data are summarized in Table 1. For the molecular characterization of *snap25s*, hatchery-reared juvenile chum salmon were used, which were provided by a commercial hatchery (Oshima Salmon Propagation Association) in April 2015. Fish were immobilized in carbonated water, and then the olfactory organ (i.e., olfactory rosette [OR]) and brain were dissected. The olfactory bulb (OB) and telencephalon (T) were dissected to examine changes in *snap25s* expression levels in the olfactory center during the seaward migration, feeding, and homeward migration. For analysis of expression levels in each brain region, brains were dissected into the olfactory center (mixture of OB and T), midbrain (mixture of optic tectum [OT] and thalamus-hypothalamus [T/H]) and hindbrain (mixture of cerebellum [C] and medulla oblongata [MO]) in post-seaward juveniles, and were dissected into OR, olfactory nerve (ON), OB, T, OT, T/H, C, MO, pituitary (P) in post-upriver mature fish. Tissues were immediately immersed in RNAlater (Ambion) and stored at -30°C until RNA extraction.

2.2. RNA preparation

Total RNA was prepared from the olfactory center, OR, and the other brain regions at several life stages of chum salmon using an acid guanidium thiocyanate-phenol-chloroform extraction method with ISOGEN (Nippon Gene) in accordance with the manufacturer's instructions. RNA purification was carried using NucleoSpin RNA clean-up (Macherey-Nagel). The total RNA concentration was measured using a NanoDrop 1000

Spectrophotometer (Thermo Scientific) by checking the A260/A280 ratio in order to confirm the purity of samples. To prepare the PCR template used for TA-cloning, poly $(A)^+$ RNA was isolated using oligo (dT)-Latex beads (Oligotex-dT30<super>; Takara) from total RNA from pooled juvenile olfactory centers dissected from 30 individuals (mean ± standard error, fork length: 53.8 ± 0.54 mm, body weight: 1.17 ± 0.04 g) to be utilized for molecular cloning.

2.3. Oligonucleotides

Oligonucleotides used as PCR primers are shown in Table 2. The SNAP-25 primer set was designed with reference to Atlantic salmon *snap25a* (GenBank accession no. **NM00173949.1**) for cloning the exon 5-corresponding region. This primer set was used to obtain sequence information in order to design the real-time quantitative PCR (qPCR) primers and the plasmid standards for qPCR. The SNAP-25 5'-untranslated region (UTR), SNAP-25 3'-UTR, and SNAP-25 exon 5a primers were also designed with reference to the Atlantic salmon *snap25a* sequence and our obtained sequence of partial chum salmon *snap25a*. To obtain the complete coding sequences (CDSs) of *snap25a* and *snap25b*, RT-PCR were conducted using the following primer sets; SNAP-25 5'-UTR-F and SNAP-25-R, SNAP-25-F and SNAP-25 3'-UTR-R, and SNAP-25 exon 5a-F and SNAP-25 3'-UTR-R.

2.4. Cloning of a salmon SNAP-25s cDNA

Briefly, poly $(A)^+$ RNA was reverse transcribed using a random primer (Random Primer $pd(N)_6$, Takara) and PrimeScript Reverse Transcriptase (Takara) with RNase inhibitor (RNase OUT, Invitrogen) in accordance with the manufacturer's instructions. PCR was performed using the following conditions, first cycle, denaturation at 94°C for 3 min, 35 cycles of incubation; 30 s at 94°C, 30 s at primer adapted temperature (61°C, 66.5°C, or 68°C) and 1 min at 72°C, and extension at 72°C for 10 min using GeneAmp PCR System 9700 (Applied Biosystems). The amplicon was rapidly T-A ligated into a pCRII TOPO vector (TOPO TA Cloning Kit, Invitrogen) and sequenced. Based on the sequence information of the partial CDS of chum salmon SNAP-25s (445 bp), qPCR primers (sSNAP-25a-F and -R, sSNAP-25b-F and -R) were designed specifically for the two SNAP-25 isoforms. These qPCR primers corresponded to the alternative spliced exon 5 regions of the *snap25* gene to avoid cross priming, and the specific amplification was confirmed by RT-PCR and 1.5% agarose gel electrophoresis. RT-PCR primers were also designed whose amplicons corresponded to nucleotide positions 87–681 and (SNAP-25 5'-UTR-F and SNAP-25-R; 495 bp) and nucleotide positions 237–821 (SNAP-25-F and SNAP-25 3'-UTR-R; 469 bp) of Atlantic salmon *snap25a* (**NM00173949.1**) including the 5'-UTR and 3'-UTR regions, respectively. A forward primer corresponding to exon 5a of chum salmon *snap25* was

designed with reference to the partial CDS of chum salmon *snap25a* (445 bp, described previously) to obtain a sequence including the 3'-UTR region (SNAP-25 exon 5a-F and SNAP-25 3'-UTR-R; 485 bp). These primers were introduced to obtain the complete CDS sequence information of salmon SNAP-25s. The resulting plasmid DNAs containing *snap25a* and *snap25b* sequences were extracted and purified using a PureYield Plasmid Miniprep System (Promega) and sequenced. For the preparations of qPCR plasmid standards, two clones (insert size; 445 bp, see above) were alkaline-denatured and purified using a DNA clean-up kit NucleoSpin gDNA Clean-up (Macherey-Nagel). These clones were used to confirm the target regions for qPCR analysis by DNA sequencing, as described in the next section. The concentrations of purified plasmid solutions were measured using a Nanodrop 1000 Spectrophotometer and then prepared as plasmid standards for absolute quantification in the qPCR procedures.

2.5. DNA sequencing analysis

DNA sequencing was conducted using a PRISM 3730 DNA analyzer and BigDye Terminator v3.1 (Applied Biosystems). Nucleotide sequence alignment was conducted using a nucleotide basic local alignment tool (blastn) offered by NCBI. Two (*snap25a* and *snap25b*) chum salmon *snap25* nucleotide sequences were deposited in GenBank as accession no. **LC315096** and **LC315097**, respectively. The alignment of multiple protein sequences was performed using the ClustalW multiple sequences alignment program based on bootstrap probabilities and viewed with molecular evolutionary genetics analysis software (MEGA, ver. 6.06). Program settings are detailed in the figure legends. Human (*Homo sapiens*) *snap25a* and *snap25b* (**L19760**, **L19761**; Bark and Wilson, 1994), chicken *snap25a* and *snap25b* (**AAA49070.1**, **AAA49071.1**; Bark et al., 1993), African clawed frog (*Xenopus laevis*) *snap25a* and *snap25b* (**AF335586**, **AF335587**), spotted gar (*Lepisosteus oculatus*) *snap25a* and *snap25b* (**XM006625688.2**, **XM006625689.2**), ray *snap25* (*Torpedo marmorat*; **L22020.1**; Risinger et al., 1993a), fruit fly *snap25* (*Drosophila melanogaster*; **L22021.1**; Risinger et al., 1993a), Atlantic salmon variant X1 and X2 (**XM014203948**, **NM001173949**), and zebrafish *snap25a* and *snap25b* (**AF091593**, **AF091594**; Risinger et al., 1998) were introduced into this alignment and sequence comparison.

2.6. qPCR

snap25s gene expression levels were quantified using a qPCR system LightCycler Nano (Roche), with intercalating dye (FastStart Essential DNA Green Master, Roche). For absolute quantification, *snap25s* pCRII-TOP10 plasmid solutions were prepared as standards using the following dilution series; 10^{-2} to 10^{-5} dilution for *snap25a* and 10^{-3} to 10^{-6} for *snap25b*. qPCR

reaction details were: 95°C for 10 min, 45 cycles three-step amplification (annealing at 60°C for *snap25a*, 65°C for *snap25b*), and then melting (60–97°C at 0.1°C per second) carried out to discriminate the specific amplifications. Primer specificities were double checked by melting analyses and 2% agarose gel electrophoresis of RT-PCR amplicons, as described previously. Every sample was duplicated to exclude outliers. Melting analyses showed specific single melting peaks with each primer sets; *snap25a* was approximately 80°C, whereas *snap25b* was approximately 87°C, which confirmed the isoform specificity and distinguishable amplification by qPCR. The quantification cycle (C_q) of all amplicons was approximately within 15–25 cycles and also ranged within the of plasmid standard C_q values, indicating they were sufficient for quantification. The experimental information from this study was documented in accordance with the minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines (Bustin et al., 2009).

2.7. Statistical analyses

snap25s expression levels were calculated as copies/total RNA (ng) for normalization among samples with different masses. All values are shown as mean ± standard error (SE). Differences were considered significant at $p < 0.05$. The Shapiro–Wilk test was used to examine the normality of the distribution of data. Tukey's honestly significant difference (HSD) test after one way ANOVA or Dunn’s multiple comparison after Kruskal–Wallis test compared the expression levels of *snap25s* among life stages; during seaward, feeding, and homing migrations using SPSS 20.0 (IBM).

3. Results

3.1. Nucleotide sequence analyses

RT-PCR amplicons of chum salmon *snap25s* were sequenced and then assembled to construct CDSs (Fig. 1). There were two types of chum salmon *snap25* isoform; *snap25a* (GenBank accession no. **LC315096**) and *snap25b* (**LC315097**). These had well-conserved amino- and carboxyl-terminal SNARE motifs that are characteristic of SNAP-25 genes. These entire sequences and their exon 5-corresponding region of the nucleotide and deduced amino acid sequences showed high similarity to other vertebrates SNAP-25 (Fig. 2). Both nucleotide sequences showed marked homology (97%). Phylogenetic analysis indicated that salmon SNAP-25a and SNAP-25b clustered within the vertebrate SNAP-25a and SNAP-25b clades, respectively, and salmon SNAP-25s was similar to the ancestral form of vertebrate SNAP-25a sequences (Fig. 3). The alignment of the two deduced amino acid sequences of salmon SNAP-25s is shown in Figure 1. Chum salmon SNAP-25a showed high similarity to higher vertebrate SNAP-25a sequences (e.g., 92% identity with human; **L19760**), while chum salmon SNAP-25b showed high similarity with that of SNAP-25b sequences (e.g., 91% identity with human; **L19761**). These chum salmon isoforms had well conserved primary structures of exon 5a or 5b, especially the cysteine-rich domain, which was described in early reports of characterized vertebrate SNAP-25 isoforms (e.g., Bark 1993; Bark and Wilson 1994; Fig. 2). Therefore, chum salmon *snap25a* and *snap25b* were identified as orthologs of higher vertebrate *snap25a* and *snap25b*, respectively.

3.2. Distributions of snap25s expression in salmon brain

snap25a was expressed in all brain regions of post-seaward juvenile chum salmon, but with no significant differences among regions (Fig. 4A). *snap25b* was expressed significantly more highly in the hind-brain than the olfactory center ($p < 0.05$, Fig. 4B). *snap25a* expression levels in all regions were 10^4-fold higher than those of *snap25b*. In post-upriver mature males, *snap25a* was expressed in all regions, at significantly higher levels in the T compared with the OR, ON, and P ($p < 0.05$), and the OB and T/H also showed significantly higher levels compared with the OR ($p < 0.05$; Fig. 5A). In post-upriver mature females, the *snap25a* expression pattern was similar to that of males (Fig. 5B). *snap25b* in mature males was expressed at significantly higher levels in the T compared with the OR, ON, and P ($p < 0.05$; Fig. 5C). *snap25b* in mature females showed similar patterns and was expressed in all brain regions, with the highest level in the T compared with the OR, ON, and P ($p < 0.05$, Fig. 5D). Expression levels of *snap25s* showed especially low levels in the OR, ON, and P of both sexes ($p < 0.05$, Fig. 5A, 5C and 5D). Two *snap25* isoforms showed different expression intensities, with *snap25a* expressed at an approximately 10^6-fold higher level than that of

snap25b, regardless of sex or brain region.

3.4. Comparison of snap25s expression levels in the olfactory center during salmon migration

Expression levels of *snap25a* in the OB were highest in seaward juveniles, at approximately 5-fold higher than that of upriver period adults ($p < 0.05$, Fig. 6A). *snap25a* in the T were expressed at significantly higher levels in the feeding migration compared with the pre- and post-seaward migrations ($p < 0.05$, Fig. 6A). Expression levels of *snap25b* in the OB showed no significant changes during salmon migration (Fig. 6B). *snap25b* levels in the T were significantly higher in feeding and post-upriver individuals and increased during upriver period ($p < 0.05$, Fig. 6B). *snap25a* expression levels in the OB and T were 10^6-fold higher than that of *snap25b* in adult salmon.

4. Discussion

The present study isolated cDNAs of two isoforms of salmon SNAP-25 expressed in the central nervous system, including the olfactory center, of several migration stages of chum salmon as the first report on salmonid *snap25* genes. These two variants of chum salmon *snap25* had well conserved SNARE motifs, confirming their identification as vertebrate *snap25* sequences (e.g., Oyler et al., 1989; Bark, 1993; Bark and Wilson, 1994). These two sequences showed amino acid substitutions restricted to the exon 5-corresponding regions, which suggested their identification as alternatively spliced isoforms of the salmon SNAP-25 mRNA. Exon 5 of *snap25* is evolutionary conserved, especially its cysteine-rich domain, which is involved in post-translational palmitoylation, resulting in the sub-functionalization of isoforms, differentiation of membranes docking, and/or vesicle release (Washbourne et al., 2001; Johansson et al., 2008) as synapse membrane SNARE proteins. The chum salmon SNAP-25s deduced amino acid sequences showed high-level homology with tetrapod SNAP-25 isoforms, which were defined in previous studies (e.g., mouse: Oyler et al., 1989; chicken: Bark, 1993; human: Bark and Wilson, 1994). These chum salmon sequences were, therefore, identified as chum salmon *snap25a* and *snap25b*, orthologs of tetrapod *snap25a* and *snap25b*, respectively.

In general, *snap25a* expression levels in the OB and T were from 10^4- to 10^6-fold higher than that of *snap25b* in chum salmon brain. This result differed from rodent brain SNAP-25s expression patterns; SNAP-25b mRNA and protein expression levels were detected at approximately 10-fold higher than that of SNAP-25a in whole brain regions as the major isoform (e.g., Johansson et al., 2008; Yamamori et al., 2011; Boshert et al., 1996). This contradiction in isoform-specific expression patterns was probably related to differences in molecular evolution (e.g., taxa-specific whole genome duplication [WGD] after tetrapod branching). The salmonid genome featured salmonid-specific WGD (e.g., Leong et al., 2010; Berthelot et al., 2014), local gene duplication (e.g., Warren et al., 2014), and later rediploidization (e.g., Lien et al., 2016; Kim et al., 2016), which brought about diversity in these functional genes. Some teleost species have tetraploid SNAP-25 genes as multiple loci, such as goldfish SNAP-25s, which were introduced as a result of WGD (Risinger et al., 1993b). Salmonid-specific WGD possibly introduced unknown SNAP-25 paralogs, and in fact Atlantic salmon multiple *snap25* transcript variants deposited predicted nucleotide sequences from genome information ("*snap25a*"-*like*: XM014144861, XM014144863, "*snap25b*"-*like*: XM014211547-50), although there were no conformations from expression analysis. The possibility could not be denied that these multiple types of *snap25* genes affected PCR efficiencies, because salmonid-specific subtypes of the respective *snap25* isoforms were not examined separately in this study. Although there was no *in vivo*

confirmation of the expression and function of teleost multiple *snap25* subtypes, differences in molecular evolution between teleosts (including salmonids) and higher vertebrate *snap25s* may affect the gene expression pattern of chum salmon *snap25s*.

Juvenile chum salmon showed *snap25s* expression in all brain regions. Salmon *snap25b* showed a partially similar expression pattern compared with rodent *snap25b* expression levels (e.g., Boschert et al. 1996, Yamamori et al., 2011), which were high in caudal brain regions (i.e., in the C and MO). This possibly reflected in juvenile CNS development requiring *snap25s* expression in the whole brain, although teleost *snap25s* functions and isoform-specific sub-functionalization are described briefly, which was an estimation based on bioinformatics (e.g., Chen and Huang, 2007). Adult salmon brains showed their highest *snap25s* expression levels in the T. *snap25s* was expressed in all brain regions, but the level was especially low in the peripheral olfactory nervous system (OR and ON) and the organ without neuronal cell body (P) (e.g., serotoninergic and dopaminergic neurons: Corio et al., 1991; and gamma amino butyric acid GABAergic neuron: Médina et al., 1994). This expression pattern was common to both sexes of post-spawning adults. The present results, which indicated that salmon *snap25s* expression is restricted to the CNS, were similar to previous studies on rodent brain SNAP-25s mRNA and protein expression levels (e.g., Boschert et al., 1996; Prescott and Chamberlain, 2011). In post-upriver adults, *snap25a* showed similar expression patterns compared with rodent *snap25a*, with high levels in rostral regions of brain (i.e., in the OB, T, and T/H) (e.g., Boschert et al., 1996, Yamamori et al., 2011). This result suggested that salmon *snap25a* is shares similar sub-functions to rodent *snap25a*. *snap25b* expression patterns were partly similar to rodent *snap25b*, being expressed in all brain regions; however, significantly higher expression in caudal regions (i.e., in the C and MO) were not detected, unlike in juveniles. One possible explanation was differences in molecular evolution among salmon and rodents, as mentioned above. Expression patterns of *snap25a* were in accordance with the small number of examples of salmon olfactory memory retrieval-related gene expression patterns, which were summarized by Ueda and colleagues (e.g., salmon type gonadotropin releasing hormone sGnRH; NMDA receptor subunit gene *nr1*: Ueda et al., 2016). In brief, synapses distributed in the adult salmon T and T/H remain plastic. This was supported by a report indicating that adult zebrafish T remains in a niche that is involved with adult neurogenesis-associated sensory-specific modulation of the CNS (Grandel and Brand 2013; Lindsey et al., 2014). On the other hand, SNAP-25 antisense oligonucleotides inhibited axonal growth in rat cortical neurons (Osen-Sand et al., 1993), and SNAP-25b expression levels associated with axon outgrowth in Schlegel's Japanese gecko (*Gekko japonicus*) spinal cord regeneration (Wang et al., 2012). Salmon *snap25s* expression levels in the T and T/H possibly reflected sensory-specific modulation or regeneration of the

CNS during homeward migration, not only olfactory but also other imprinted cues. It was described previously that memory retrieval in homing salmon is partly regulated by the sGnRH neuroendocrine system (Ueda et al., 2016), although the main effects of sGnRH regulation are the initiation of homing behavior and sexual maturation (Onuma et al., 2010). sGnRH protein and sGnRH precursor mRNA expression is distributed in the ON, OB, T, preoptic area, and connecting regions of all of them in both sexes of homing mature chum salmon (Kudo et al., 1996). In mammals, female rat GnRH neuroendocrine cells express the mRNAs and proteins for SNAP-25 and Munc-18 as major SNARE complex proteins (Weiss et al., 2007). Therefore, the similarity of expression patterns of homing salmon brain *snap25s* and sGnRH suggests that salmon *snap25s* are associated with sGnRH release at the synapse terminal of homing salmon.

The present results suggested that high expression levels of *snap25a* in the OB were involved in synaptogenesis in seaward juveniles, and an increase in *snap25b* expression in the T during the upriver period was involved in the olfactory memory retrieval to natal odors by synaptic plasticity in higher olfactory center, as previously showed in adult zebrafish T (Grandel and Brand 2013; Lindsey et al., 2014). Although expressed at a low level, *snap25b* in the T showed similar expression changes to previously reported NMDA-type glutamate receptor *nr1* as imprinting-related genes during seaward migration (Yu et al., 2014; Ueda et al., 2016). It was suggested that SNARE components and NMDA receptors function cooperatively in vesicle trafficking to the postsynaptic plasma membrane and facilitate synaptic plasticity in rat hippocampal post-synaptic spines (Hussain et al., 2016). It is reasonable that high *snap25s* expression reflected high levels of synaptic plasticity and indicated synaptogenesis (e.g., Catsicas et al., 1991) of newborn neurons during the early life stages of salmon. This assumption was supported by the dramatic proliferation of olfactory sensory neurons in juvenile chum salmon, whereas adults showed slower increases accompanied by somatic growth (Kudo et al., 2009). Similarly, multiple olfactory receptor mRNA expression in the Atlantic salmon olfactory epithelium was significantly higher at the smolt stage (i.e., seaward period), whereas it was low in adults (Johnstone et al., 2011). Therefore, juvenile *snap25a* expression levels associated with synaptic plasticity in the OB reflected the proliferation of olfactory sensory neurons. Neurotransmitter (e.g., serotonin, dopamine, glutamine, and gamma-amino butyric acid) concentrations in synaptic transmission in the brain increased during the parr–smolt transformation of coho salmon juveniles (Ebbesson et al., 1992, 1996, 2003). As is well known, these neurotransmitters are released into the synaptic cleft by vesicle exocytosis, for which SNARE complexes provide regulation (Lin and Scheller, 2000). It is a reasonable assumption that high *snap25s* expression levels are related to the high levels of neurotransmitters exocytosis in synapses distributed to the OB of

chum salmon juvenile. For these reasons, three components of neurotransmission (neurotransmitters, SNARE complexes, and neurotransmitter receptors) are activated and related to olfactory imprinting reciprocally or cooperatively in the juvenile salmon brain. *snap25a* in the olfactory center decreased or remained at low levels during homeward migration. These results suggested that existing olfactory systems were involved in discriminating the natal site, not newly generated neurons or synapses.

In conclusion, we performed molecular cloning and verified the expression of salmon *snap25s* in the chum salmon brain and olfactory organ. Our results are the first report of *snap25* gene expression in the salmon brain and indicate that *snap25s* is involved in the synaptic plasticity of olfactory imprinting and/or olfactory memory retrieval in Pacific salmon. Here, we performed an initial analysis of the pre-synaptic functional molecular component SNAP-25, and our findings provide fundamental knowledge for future olfactory imprinting studies.

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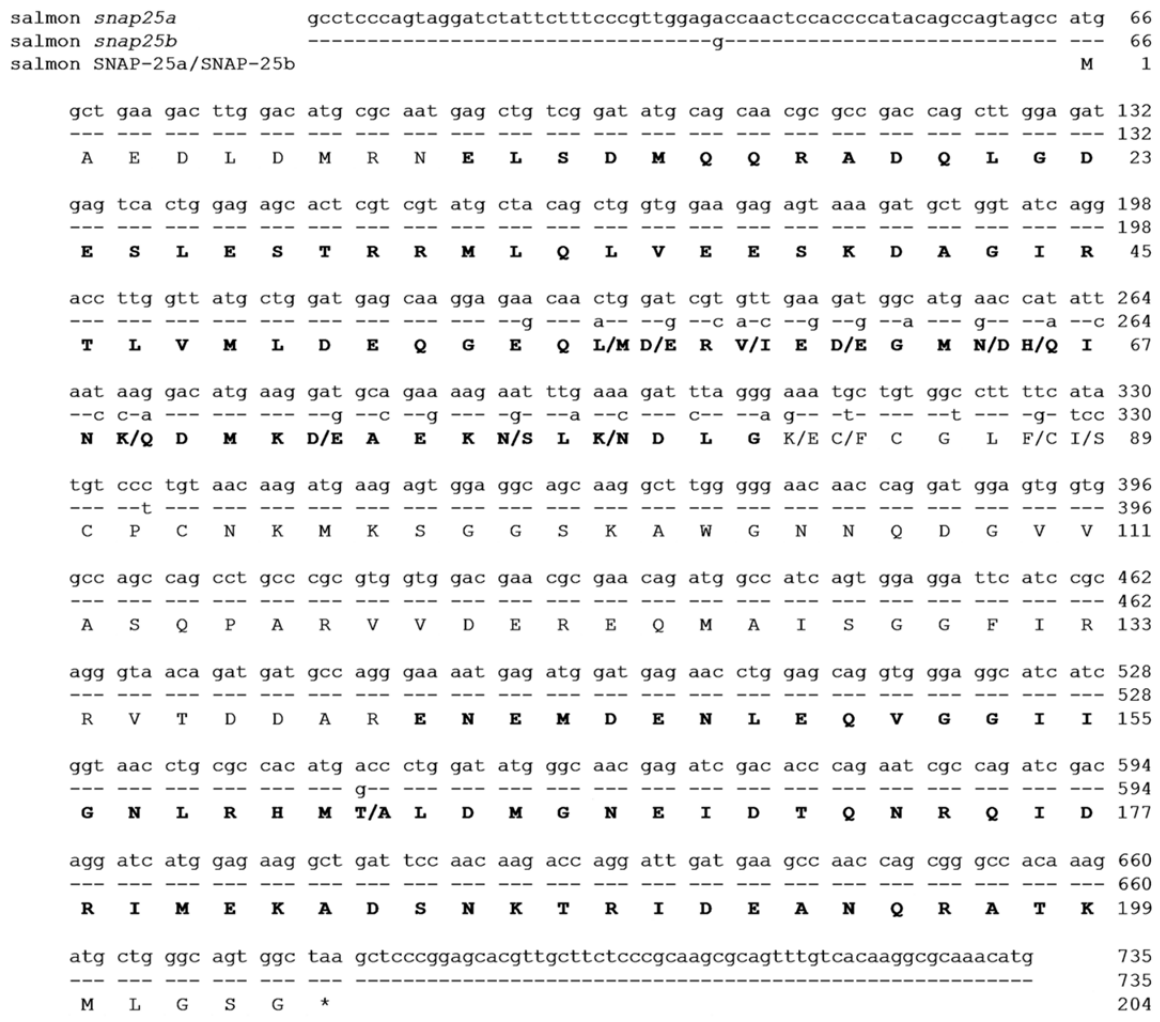


Fig. 1. The nucleotide and deduced amino acid sequences of chum salmon, *Oncorhynchus keta*, SNAP-25a and SNAP-25b. Nucleotide acids identical between salmon *snap25a* (**LC315096**) and *snap25b* (**LC315097**) are shown by dashes (-). Bold letters in the amino acid sequences (positions 10–82 and 141–199) indicate the N-terminal and C-terminal SNARE motifs, respectively.

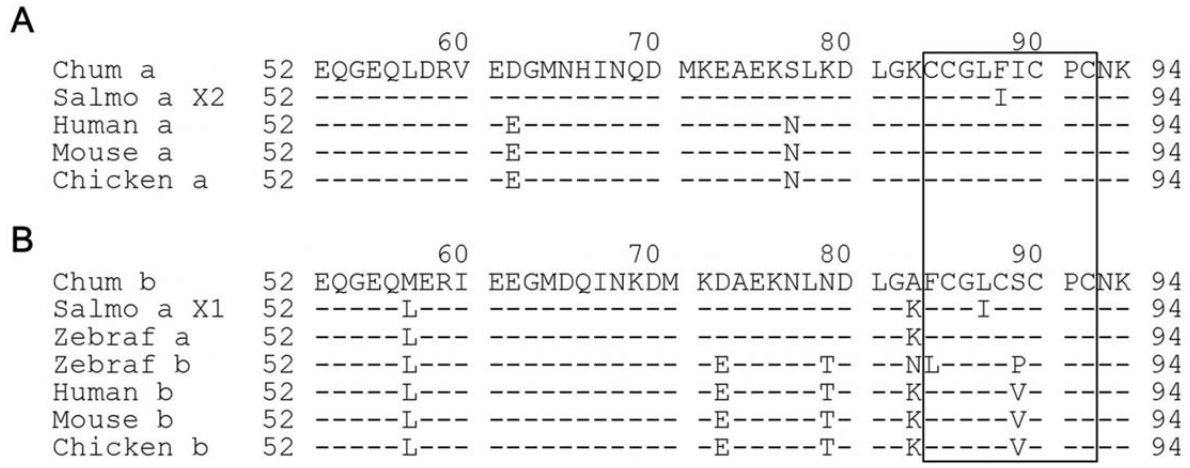


Fig. 2. Alignment of amino acid sequences of exon 5a (A) and 5b (B) corresponding regions of SNAP-25 are shown, with deduced amino acid sequences of chum salmon ("Chum"), *Oncorhynchus keta*, isoform-a (**LC315096**) and isoform-b (**LC315097**), and those of other vertebrates; Atlantic salmon ("Salmo"), *Salmo salar*, SNAP-25a1 (**XM014144861.1**) and -25a2 (**XM014144863.1**), zebrafish ("Zebraf"), *Danio rerio*, SNAP-25a (**AF091593**) and -25b (**AF091594**), human, *Homo sapiens*, SNAP-25a (**L19760**) and -25b (**L19761**), and chicken, *Gallus gallus domesticus*, SNAP-25a (**AAA49070.1**) and -25b (**AAA49071.1**). Dashed lines correspond to residues identical to chum SNAP-25a and b. The cysteine-rich domain, as a distinguishable feature of SNAP-25 isoforms (e.g., Bark, 1993), is boxed.

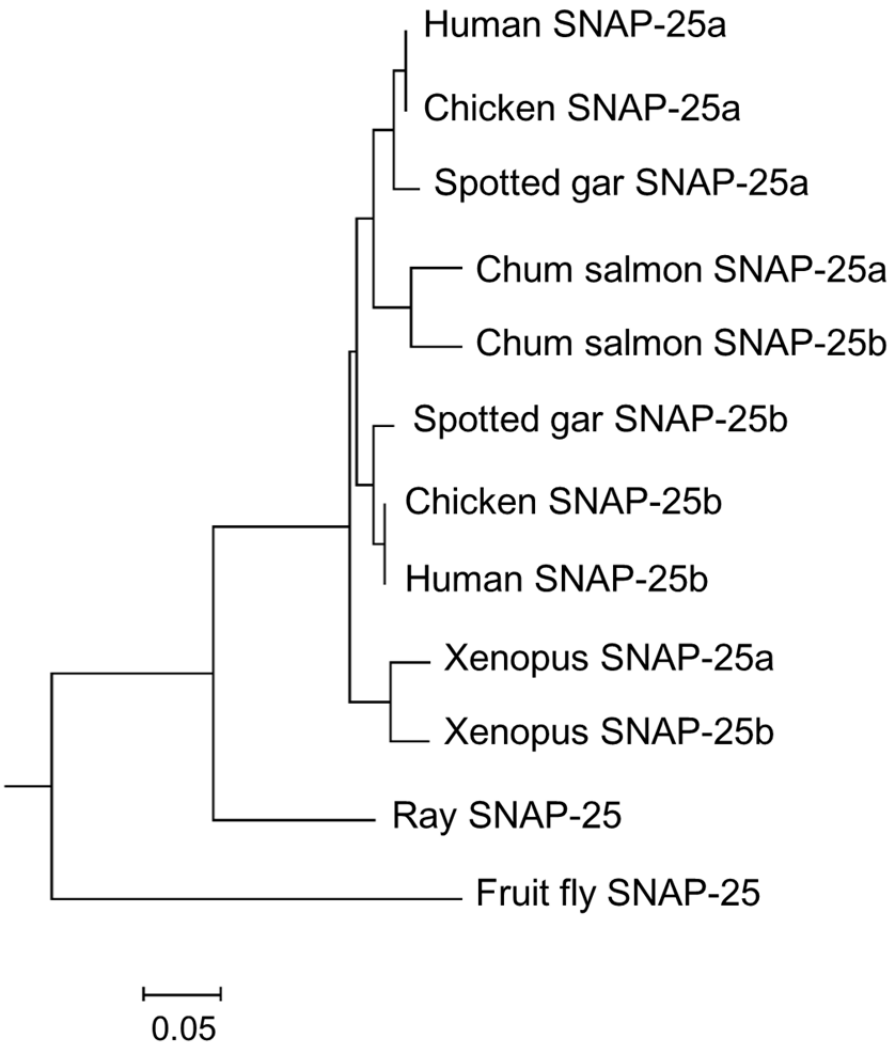


Fig. 3. Evolutionary relationships between the known SNAP-25s. This phylogenetic tree was constructed using the neighbor joining (N-J) method. A comparison was made with amino acid sequences of chum salmon, *Oncorhynchus keta*, SNAP-25 isoform-a (**LC315096**) and isoform-b (**LC315097**), spotted gar, *Lepisosteus oculatus*, SNAP-25a (**XM006625688.2**) and -25b (**XM006625689.2**), ray, *Torpedo marmorata*, (**L22020.1**), African clawed frog, *Xenopus laevis*, SNAP-25a (**AF335586**) and-25b (**AF335587**), chicken, *Gallus gallus domesticus*, SNAP-25a and -25b (see Fig. 1.), human, *Homo sapiens*, SNAP-25a and -25b (see Fig. 1), and fruit fly, *Drosophila melanogaster*, (**L22021.1**). The scale bar shows relative genetic distances.

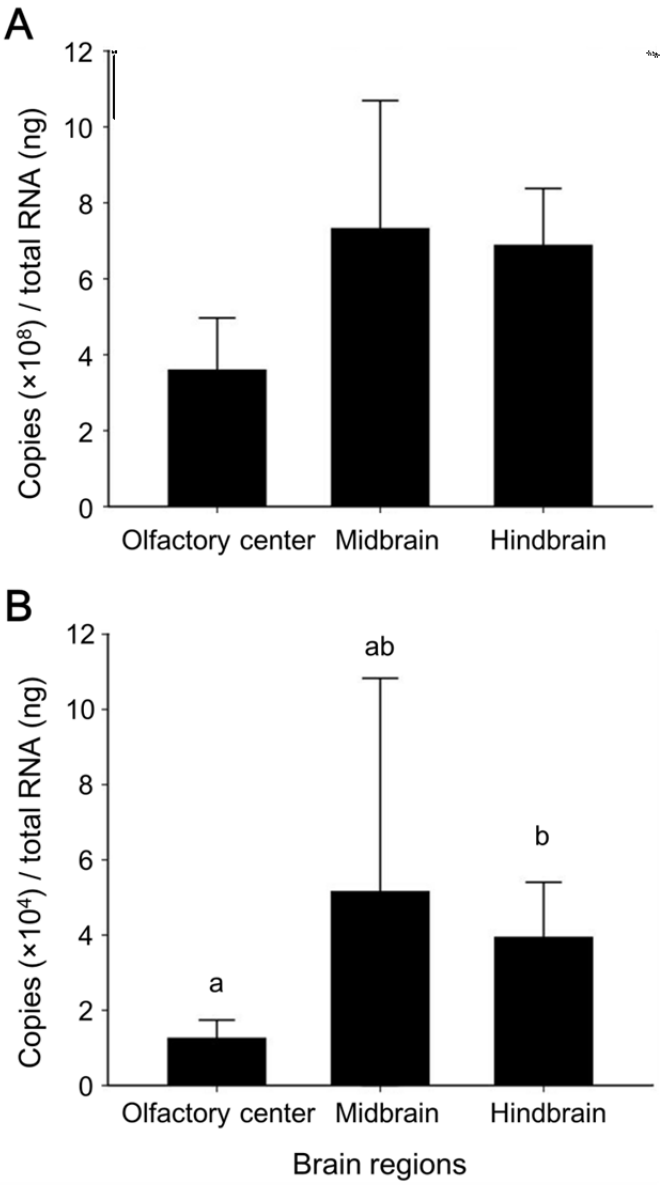


Fig. 4. Expression of synaptosome-associated protein 25 isoform-a (*snap25a*: A) and isoform-b (*snap25b*: B) in the olfactory center (mixture of olfactory bulb and telencephalon), midbrain (mixture of optic tectum, thalamus, and hypothalamus), and hindbrain (mixture of cerebellum and medulla oblongata) of post-seaward juveniles using quantitative real-time PCR. Values represent mean ± standard error (n=5-7) of copies par sample RNA (ng). No significant differences were shown among regions by ANOVA ($p > 0.05$, Fig. 4A). Symbols sharing the same letter are not significantly different from each other by Kruskal-Wallis test followed post hoc Dunn's multiple comparison test ($p < 0.05$, Fig. 4B).

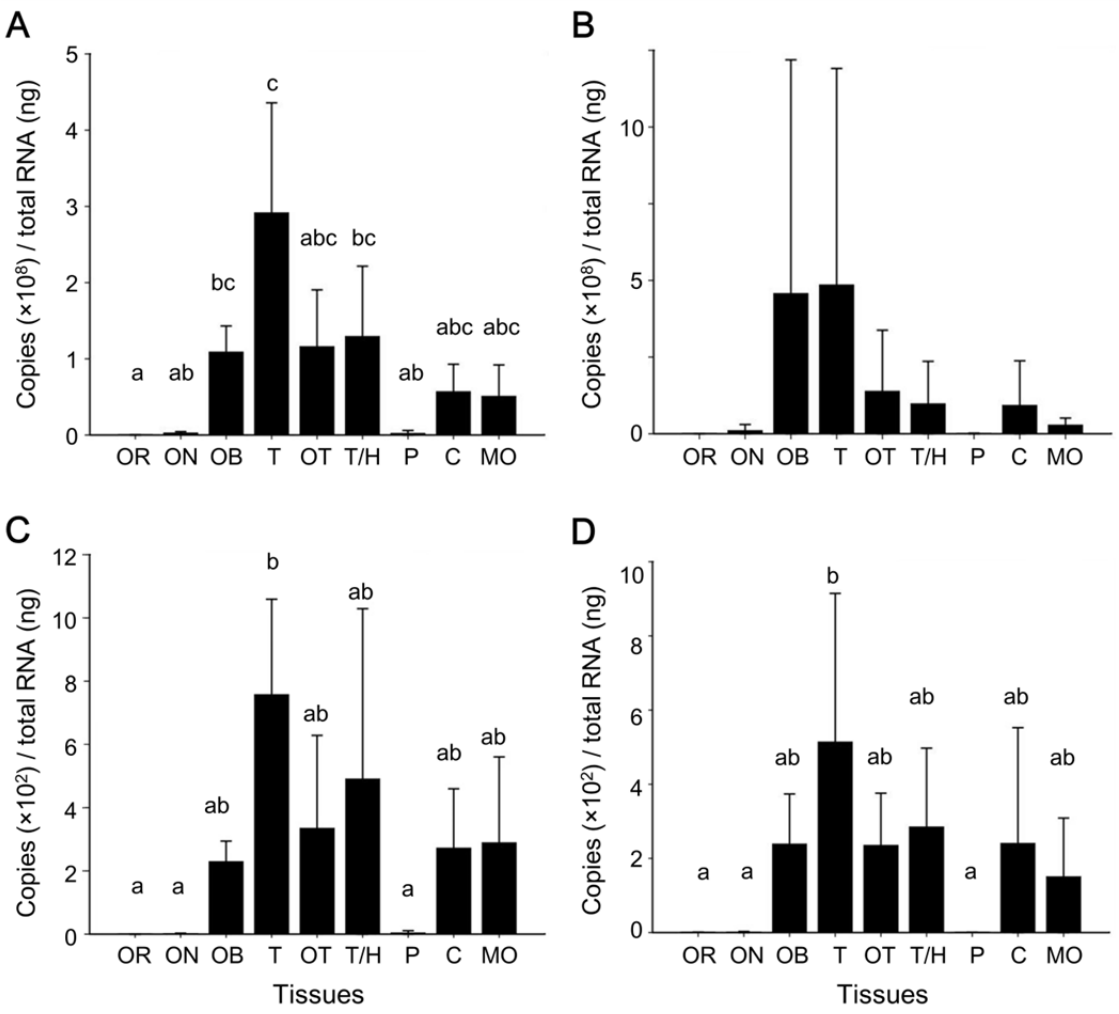


Fig. 5. Expression of SNAP25 isoform-a (*snap25a*: A, B) and isoform-b (*snap25b*: C, D) in the olfactory organ and brain of post-upriver male (A, C) and female (B, D) chum salmon using qPCR. C, cerebellum; MO, medulla oblongata; OB, olfactory bulb; ON, olfactory nerve; OR, olfactory rosette; OT, optic tectum; P, pituitary; T, telencephalon; T/H, mixture of thalamus and hypothalamus. Values represent mean ± standard error (male: n=5, female: n=3) copies per sample RNA (ng). Symbols sharing the same letter are not significantly different from each other ($p < 0.05$, Kruskal-Wallis test followed post hoc Dunn's multiple comparison test: A and B, one-way ANOVA followed post hoc Tukey's HSD: C and D).

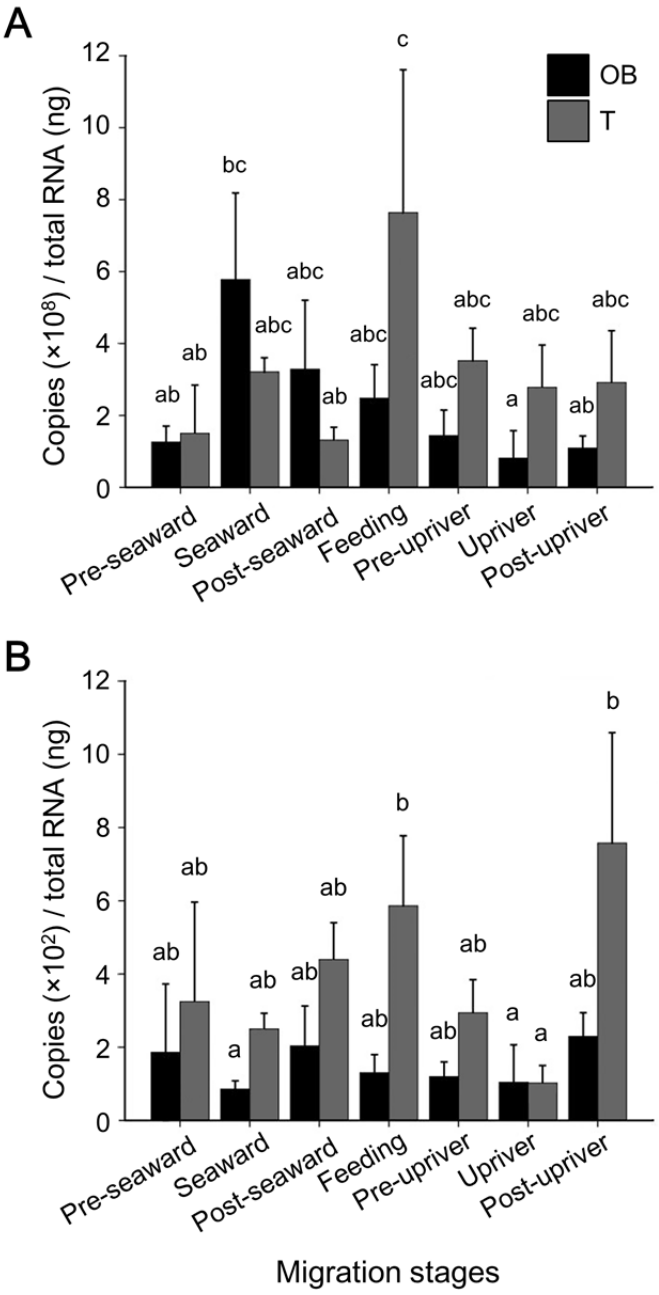


Fig. 6. Expression of synaptosome-associated protein 25 isoform-a (*snap25a*: A) and isoform-b (*snap25b*: B) in the olfactory bulb (OB: black column) and telencephalon (T: gray column) during seaward and homeward migration using qPCR. Values represent mean ± standard error (n=5–6) copies per sample RNA (ng). Symbols sharing the same letter are not significantly different from each other ($p < 0.05$, Kruskal–Wallis test followed post hoc Dunn's multiple comparison test).

Table 1. Data sets of the seaward, feeding and homeward migrating juveniles and adult chum salmon, *Oncorhynchus keta*, in this study, respectively. Values of fork length (mm) and body weight (g) are expressed as mean ± standard error. Date and location of capturing, sampling gear and sample size (individuals) are also shown.

Stage	Fork length (mm)	Body weight (g)	Number of samples	Date	Location or source	Gear
Pre-seaward	39.3 ± 0.80	0.42 ± 0.03	5	22-Mach-2017	Upper reaches of Yurappu River	Electric shocker
Seaward	52.7 ± 2.17	1.07 ± 0.12	5	20-Apr-2017	Lower reaches of Yurappu River	Electric shocker
Post-seaward	51.9 ± 1.94	0.97 ± 0.13	12	11-May-2016, 5-May-2017	Seawater rearing in laboratory	—
Feeding	541 ± 20.5	1751 ± 263.8	6	15 to 18-May-2017	Northwest Pacific Ocean (43°N, 155°E)	Angling
Pre-upriver	763 ± 22.1	4432 ± 452.7	5	23-Oct-2016	Coastal waters off Yakumo	Set net
Upriver	764 ± 13.3	4793 ± 283.8	5	10-Nov-2016	Lower reaches of Yurappu River	Electric shocker
Post-upriver	704 ± 17.6	3332 ± 389.3	8	24-Nov-2016	Lower reaches of Yurappu River	Electric shocker

Table 2. Primer sets used in RT-PCR for sequencing, and quantitative real-time PCR of chum salmon, *Oncorhynchus keta*, synaptosome-associated protein 25 isoform-a (*snap25a*) and isoform-b (*snap25b*).

Primer name	Sequences	Source
SNAP-25-F	5'-CGTCGTATGCTACAGCTGGT-3'	NM00173949.1
SNAP-25-R	5'-TGTCGATCTGGCGATTCTGG-3'	NM00173949.1
SNAP-25 5'-UTR-F	5'-GCCTCCCAGTAGGATCTATTCTTTC-3'	NM00173949.1
SNAP-25 3'-UTR-R	5'-CATGTTTGCGCCTTGTGACAAA-3'	NM00173949.1
SNAP-25 exon5a-F	5'-CCAAGACATGAAGGAGGCCGAG-3'	This study
sSNAP-25a-F	5'-ATCGTGTTGAAGATGGCATGAA-3'	This study
sSNAP-25a-R	5'-CTTGCTGCCTCCACTCTTCATC-3'	This study
sSNAP-25b-F	5'-CTGTGGTCTTTGCTCCTGTCCT-3'	This study
sSNAP-25b-R	5'-TCCTCCACTGATGGCCATCTGT-3'	This study