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Effect of salinity changes on olfactory memory-related genes and hormones in adult chum salmon *Oncorhynchus keta*

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ABSTRACT

Studies of memory formation have recently concentrated on the possible role of N-methyl-D-aspartate receptors (NRs). We examined changes in the expression of three NRs (NR1, NR2B, and NR2C), olfactory receptor (OR), and adrenocorticotropic hormone (ACTH) in chum salmon *Oncorhynchus keta* using quantitative polymerase chain reaction (QPCR) during salinity change (seawater → 50% seawater → freshwater). NRs were significantly detected in the diencephalon and telencephalon and OR was significantly detected in the olfactory epithelium. The expression of NRs, OR, and ACTH increased after the transition to freshwater. We also determined that treatment with MK-801, an antagonist of NRs, decreased NRs in telencephalon cells. In addition, a reduction in salinity was associated with increased levels of dopamine, ACTH, and cortisol (*in vivo*). Reductions in salinity evidently caused NRs and OR to increase the expression of cortisol and dopamine. We concluded that memory capacity and olfactory imprinting of salmon is related to the salinity of the environment during the migration to spawning sites. Furthermore, salinity affects the memory/imprinting and olfactory abilities, and cortisol and dopamine is also related with olfactory-related memories during migration.

1. Introduction

Salmon are long-distance migratory fish that are widely distributed from countries of the North Pacific Rim and Asia (Groot and Margolis, 1991). Pacific salmon *Oncorhynchus* are anadromous fish that spend most of their lives rearing in the ocean before returning to freshwater to spawn (Groot and Margolis, 1991; Salo, 1991; Ueda, 2011). There are seven species of Pacific salmon: sockeye salmon (*Oncorhynchus nerka*), pink salmon (*Oncorhynchus gorbuscha*), chum salmon (*Oncorhynchus keta*), chinook salmon (*Oncorhynchus tshawytscha*), coho salmon (*Oncorhynchus kisutch*), masu salmon (*Oncorhynchus masou*), and amago/biwamasu salmon (*Oncorhynchus thodurus*). Among Pacific salmon in Korea, most chum and masu return to their natal river (Machidori and Katou, 1984; Quinn, 2005; Jeon et al., 2011).

Pacific salmon travel thousands of kilometers to the upper portions of river to reproduce (Quinn, 2005) Newly hatched salmon fry possess knowledge of certain environmental features of the river imprinted in their nervous systems, allowing them to recognize these environmental factors when they migrate upstream as adults (Ueda, 2011). Recent studies have reported on the neuroendocrine aspects of hormones involved in migratory and homing behavior, including catadromous migration of salmon fry and homing migration of adult salmon (Tipsmark et al., 2010; Johnstone et al., 2012; Choi et al., 2014). The exact mechanism that guides the salmon's returning behavior remains unclear. The mechanisms of salmonid homing are not completely understood, but it is known that adult salmon continuously utilize two of their primary sensory systems: olfaction and vision; and environment change (salinity, magnetic, and rheotaxis) during homing (Ueda et al., 1995; Dittman and Quinn, 1996; Putman et al., 2014). It is known that sensitivity to changes in the olfactory neurons is significantly associated with a mechanism for storing the knowledge of these streams (Hasler and Scholz, 1983; Johnstone et al., 2012).

Olfactory memory studies have recently focused on the possible role of N-methyl-D-aspartate receptors (NRs), which are glutamate receptors, in the formation of memory (Xia et al., 2005; Tzeng et al., 2007; Sison and Gerlai, 2011). The study of NRs has focused on long-term memory, which is responsible for learning and memory control in the brain (Kinoshita et al., 2004, 2005). NRs regulate immediate–early gene expression, improve the long-term storage capacity of the brain, and increase the expression of genes associated with olfactory imprinting during parr-smolt transformation (Fukaya, 1999; Kinoshita et al., 2004, 2005). NRs are heteromers composed of two NR subunits, NR1 and NR2, which in turn include one NR1 and four NR2s (NR2A-D) (Cox et al., 2005; Kinoshita et al., 2005).

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Teleosts have a very well-developed olfactory sense that they use to find food, detect predators, select partners, and communicate with one another (Hara, 1994; Sorensen et al., 1995). Specially, salmon detect odorants through the activity of olfactory receptors (ORs); seven transmembrane G-protein coupled receptors that are expressed by sensory neurons of the olfactory epithelium (Buck and Axel, 1991; Alioto and Ngai, 2005). Four distinct classes of vertebrate ORs have been identified to date: main olfactory receptors (mORs; appear to highly conserved among species and expressed in ciliated olfactory receptor cells), trace amine-associated receptors (TAARs), vomeronasal type 1 receptors (V1Rs) and vomeronasal type 2 receptors (V2Rs; Mombaerts, 2004; Johnson and Banks, 2011). The olfactory sensory system of fish affects their entire life cycle and performs an essential role in biological processes to detect information about the outside environment. The olfactory-related information is delivered to the brain by the ORs in the olfactory epithelium (Cao et al., 1998; Saraiva and Korsching, 2007). In salmonids, ORs play an important role in imprinting in connection with the NRs (Johnstone et al., 2012).

Salinity change is one of the most important direct environmental factors that influence spawning migration. Also, salinity preferences have been suggested to play a role in orientation during home-stream migration in sea-run Pacific salmon (Dittman and Quinn, 1996). During this process, increased cortisol levels enhance the ability to adapt to changes in the environment, which allows the fish to maintain the balance of water and electrolytes *in vivo* and helps control the plasma osmolality (Mommsen et al., 1999). Corticotropic-releasing hormone is the key factor in the hypothalamus–pituitary–interrenal (HPI) axis and causes the release of adrenocorticotropic hormone (ACTH), which acts on the adrenal cortex to release cortisol (Huising et al., 2004). In addition, the increase in plasma cortisol levels following the activation of the HPI axis is probably required for central nervous system activation and higher blood glucose concentrations (Bernier et al., 1999; Doyon et al., 2006).

In a recent study, Choi et al. (2014) reported that levels of plasma cortisol in female chum salmon increased during adaptation to artificial seawater desalination, as did plasma estradiol-17β and vitellogenin, which are involved in maturity.

In mammals, dopamine plays an essential role in movement, learning, behavior, and sensory understanding. It functions in the transfer of information through the olfactory system to the brain (Hsia et al., 1999; Davila et al., 2003). The dopamine neurotransmitter increases in fish in response to salinity changes (Péqueux, 1995). In addition, increased dopamine levels are found in the brains of salmon during spawning migration, and dopamine is reported to be associated with memory formation and imprinting (Weltzien et al., 2006).

In the present study, salmon experienced the transition from saline water (SW) to 50% SW to freshwater (FW), corresponding to the changes in salinity that adult female chum salmon experience as they travel upstream from coastal areas to spawning areas. We determined the effects of salinity change, the most important factor affecting memory formation and imprinting during migration, by measuring the expression of NRs and OR (through mRNA and proteins) and plasma dopamine. In addition, we measured the expression of ACTH mRNA and plasma levels of ACTH and cortisol on the HPI axis. We also investigated changes in NRs, memory formation-related genes, in the telencephalon and OR, an olfactory imprinting-related gene, in the olfactory epithelium. Specifically, we analyzed changes in the mRNA expression of NRs and OR by applying MK-801 (dizocilpine), an antagonist of NR and OR, during salinity changes *in vivo* and *in vitro*.

2. Material and methods

2.1. Experimental fish

Mature female chum salmon (*O. keta*, length = 71.4 ± 8.4 cm, mass = 2.82 ± 0.37 kg, gondosomatic index [gonad weight/body weight] = 18.3 ± 3.8) were collected from the coastal area of the East Sea, Yangyang, Korea, and were transported to the Marine Biology Center for Research & Education at Gangnung-Wonju National University, Gangnung, Korea. Fish were maintained in four 40 L tanks for the duration of the experiment (3 days).

The transfer of chum salmon from SW (35 psu) to FW (0 psu) followed a specific procedure. The salmon were acclimated in a square tank filled with SW, following which spring water was poured into the tank to give a concentration of 50% SW (17.5 psu); fish were maintained in this water for 24 h, after which more spring water was added to completely dilute the tank water to FW, in which the fish were held for a further 24 h. We have used spring water in Namdaecheon River (Gangnung, Korea; pH 6.4–6.8, dissolved oxygen 9.07–11.02 mg/L). The water temperature was maintained at 18.5 ± 0.5 °C. No fish died during the experimental period.

2.2. Sampling

The fish were anesthetized in 0.005% eugenol (4-allyl-2-methoxyphenol) and tissues were selected for analysis from 5 randomly selected fish for each salinity (SW, 50% SW, and FW). Their brains and pituitary glands were frozen in liquid nitrogen and stored at −80 °C until total RNA extraction was performed. Blood was taken from the caudal vasculature using a 3-mL heparinized syringe. After centrifugation (10,000 ×*g*, 4 °C, 5 min), the plasma was stored at −80 °C before analysis.

2.3. Brain incubation

After the fish were anesthetized, their telencephalon and olfactory epithelium were dissected and placed in an ice-cold medium (pH 7.5) composed of 25 mM HEPES, 4 mM $NaHCO_3$, 0.3% BSA, 0.1% collagenase, 0.25 mg/mL fungizone, and RPMI medium containing antibiotics (100 U/L penicillin and 100 mg/L streptomycin; penicillin–streptomycin, Gibco, Carlsbad, CA, USA). A scalpel was used to cut each telencephalon and olfactory epithelium into 1–3-mm^3 pieces. The pieces were weighed, placed in a 24-well culture plate (SPL Life Science, Gyeonggi, Korea) containing 1 mL of medium, and incubated at 20 ± 1 °C in an incubator for 1 day. Although explants occasionally adhered to the bottom of the wells, they typically remained unattached during culture. The cultured intestine was sampled at 24-h intervals during the transition of fish from SW to FW; each sample was centrifuged (20 °C, 10,000 ×*g*, 15 s), and the supernatant was removed and stored at −80 °C until required for RNA extraction.

To investigate the relationship between salinity changes and NRs and OR during the transition from the ocean to rivers, and to understand the role of MK-801, an antagonist of NR, on chum salmon, MK-801 (2 and 20 μM) was added to cultured telencephalon and olfactory epithelium (*in vitro*). MK-801 (M107; Sigma, St. Louis, MO, USA) dissolved in 0.9% physiology saline was added to the culture medium in a ratio of 1/1000 (v/v), and the specified concentrations of MK-801 (2 and 20 μM) were added. Each sample was centrifuged (20 °C, 10,000 ×*g*, 15 s), and then the supernatant was removed and stored at −80 °C until RNA extraction.

2.4. Tissue distribution of NR mRNAs

To examine the tissue distribution of the mRNA of select NR subunits (NR1, NR2B, and NR2C) and OR, total RNA was extracted from the pituitary, diencephalon, optic tectum, telencephalon, cerebellum, olfactory nerve, olfactory epithelium, and olfactory bulb, as previously described. Total RNA was extracted from the tissues using Tri-Reagent (MRC, Cincinnati, OH, USA). Reverse transcription (RT) was performed of cDNA using M-MLV reverse transcriptase (Promega, OH, USA) according to the manufacturer's instructions. The following RT-PCR primers were designed with reference to the known sequences of the chum salmon (GenBank accession numbers: NR1, JQ924060; NR2B,

KM509062; NR2C, KF595125; OR, FJ613852, β-actin, AB032464): NR1 forward (5′-CAG GAG ACT AAC TTA ACT GC-3′) and reverse (5′-TCA CTA TCT TTA GTC TTG AG-3′) primers; NR2B forward (5′-GAG GAG CGC TCA GAG GTC ATT G-3′) and reverse (5′-CCT GAG ACC CTG GAG TGA-3′) primers; NR2C forward (5′-AAC GGC ATG TTC AAG GTG AT-3′) and reverse (5′-ATG ACA GCG AAG GCC C-3′) primers; OR forward (5′-GTT TGT CAT CGT TGG GTT CC-3′) and reverse (5′-TGA CCA CTG CAG GTA GAA AG-3′) primers, and β-actin forward (5′-GGA CCT GTA TGC CAA CAC TG-3′) and reverse (5′-TGA TCT CCT TCT GCA TCC TG-3′) primers. PCR amplification was performed with specific primer sets with the ex × Taq (RR001A, TaKaRa, Tokyo, Japan). PCR was carried out as follows: initial denaturation at 95 °C for 2 min; then 40 cycles consisting of denaturation at 95 °C for 20 s, annealing at 56 °C for 40 s, and extension at 72 °C for 1 min; followed by 7 min at 72 °C for the final extension. Amplification of β-actin mRNA was used to verify the quality of the RT products, using a primer set specific for chum salmon β-actin cDNA. The amplified PCR products were electrophoresed on 1% agarose gels, detected by staining with ethidium bromide, and visualized by illumination with UV light.

2.5. Quantitative real-time PCR (QPCR)

We used QPCR to determine the relative expression of NR mRNA using total RNA extracted from chum salmon. Primers for QPCR were designed with reference to the known sequences of chum salmon (Table 1). Amplification was conducted using a Bio-Rad CFX96™ Real-time PCR Detection System (Bio-Rad, Hercules, CA, USA) and iQ™ SYBR Green Supermix (Bio-Rad) with the following conditions: 0.5 μL of cDNA, 0.26 μM of each primer, 0.2 mM dNTPs, SYBR Green, and Taq polymerase in buffer (10 mM Tris–HCl [pH 9.0], 50 mM KCl, 1.4 mM $MgCl_2$, and 20 nM fluorescein) to a total volume of 25 μL. The QPCR process included one cycle of initial denaturation at 95 °C for 5 min 35 cycles of denaturation at 95 °C for 20 s and annealing at 55 °C for 20 s; followed by 7 min at 72 °C for the final extension. Each experimental group was run in triplicate to ensure consistency. As an internal control, experiments were duplicated with β-actin, and all data were expressed as the change with respect to the corresponding β-actin calculated threshold cycle (Ct) levels. All analyses were based on the Ct values of the PCR products.

2.6. Western blot analysis

Total protein isolated from the brains and olfactory epithelia of female chum salmon during salinity changes was extracted using a T-PER® Tissue Protein Extraction Reagent (Thermo fisher scientific, Inc., USA) according to the manufacturer's instructions, after which it was sonicated and quantified using the Bradford method (Bio-Rad). The total protein (30 μg per lane) was loaded per lane onto Mini-PROTEAN® TGX™ Gels (Bio-Rad, USA). For reference, a protein ladder (Bio-Rad, USA) was also used. Samples were electrophoresed at 180 V. The gels were immediately transferred to a 0.2 μm polyvinylidene difluoride (PVDF) membrane (Bio-Rad, USA) at 85 V for 3 min using the Trans-Blot® Turbo™ Transfer System. Thereafter, the membranes were blocked with 5% milk in TBS (pH 7.4) for 45 min and then washed in TBS. The membranes were incubated with NR1 (dilution, 1:4000; ABIN183668; Antibodies-Online), NR2B (dilution, 1:2000; AB1557P; Millipore), and OR ($G_{\alpha\ s/olf}$ [C-18]; dilution, 1:1000; SC-383; Santa Cruz Biotechnology) followed by a horseradish peroxidase conjugated anti-goat IgG secondary antibody (1:5000; Bio-Rad) for 60 min. The internal control was a β-tubulin antibody (dilution, 1:5000; ab6046, Abcam, UK), followed by a horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (1:4000; Bio-Rad) for 60 min. The bands were detected using WesternBright™ ECL (Advansta, Menlo Park, CA, USA) with a 30-s exposure and a Molecular Imager® ChemiDoc™ XRS + System (Bio-Rad). The membrane images were scanned with a high-resolution scanner, and the band density was estimated using a computer program (Image Lab™ Software, version 3.0, Bio-Rad). The ratios of the internal control (β-tubulin) to the NR subunits and OR for each concentration were calculated and plotted against the concentration of the internal control.

Table 1
Primers used for amplification of QPCR.

Genes	Primer	DNA sequences
NR1	Forward	5′-CAG GCG AAC CAG ATA TAC G-3′
	Reverse	5′-AGG ATG ACT CAC GAG GAT G-3′
MR2B	Forward	5′-CAT CCT CAT GCT GTT CGG-3′
	Reverse	5′-TGT AGA AGA CAC CTG CCA T-3′
MR2C	Forward	5′-GGA AGC ACA GAG AGG AAC A-3′
	Reverse	5′-GCA CAG CAG CGT CAT AGA-3′
OR	Forward	5′-CAT ATC GCT ATA ACA ACG CTT G-3′
	Reverse	5′-GAA CAA CTG CCA CAA TAA TAG AG-3′
ACTH	Forward	5′-CGA CTC TGA CTC TCC TCC-3′
	Reverse	5′-CAC ACT GTT CTG CTG CTC-3′
β-Actin	Forward	5′-ATC TGG CAT CAC ACC TTC TA-3′
	Reverse	5′-CTT CTC CCT GTT GGC TTT G-3′

2.7. Plasma parameter analysis

Plasma ACTH, cortisol, and dopamine levels were analyzed by immunoassay using an ELISA kit (ACTH, E15926Fh; cortisol, E12121Fh; dopamine, EQ027496FI; Cusabio Biotech, Hubei, China).

2.8. Statistical analysis

All data were analyzed using the SPSS statistical package (version 10.0; SPSS Inc., USA). One way ANOVA was followed by Tukey *post hoc* test to compare differences in the data ($p < 0.05$). Values are expressed as mean ± SE.

3. Results

3.1. Tissue distribution of NR subunit mRNAs

Fig. 1 shows the tissue-specific expression patterns of chum salmon NR subunits (NR1, NR2B, and NR2C) and OR mRNA in FW. NR mRNA was primarily detected in the whole brain and olfactory area and was significantly detected in the diencephalon and telencephalon of chum salmon. OR mRNA was primarily detected in the telencephalon and whole olfactory area and was significantly detected in the olfactory epithelium of chum salmon.

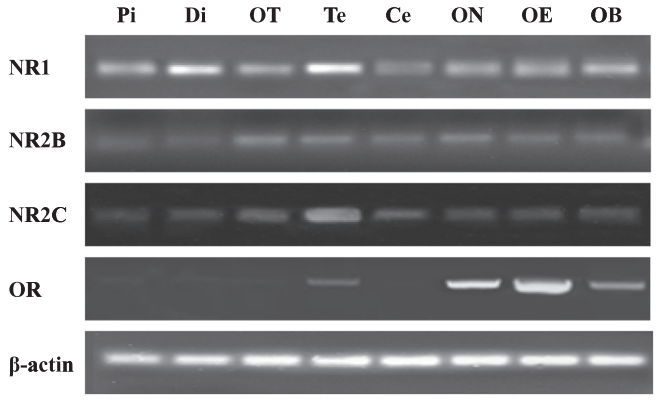

Fig. 1. Tissue distribution of chum salmon N-methyl-D-aspartate receptors (NRs) and olfactory receptor (OR). Reverse transcriptase-PCR analysis of NRs and OR transcripts in different tissues collected from March 2014, as shown in a 1.0% agarose electrophoresis gel with ethidium bromide. Pi, pituitary; Di, diencephalon; OT, optic tectum; Te, telencephalon; Ce, cerebellum; ON, olfactory nerve; OE, olfactory epithelium; and OB, olfactory bulb.

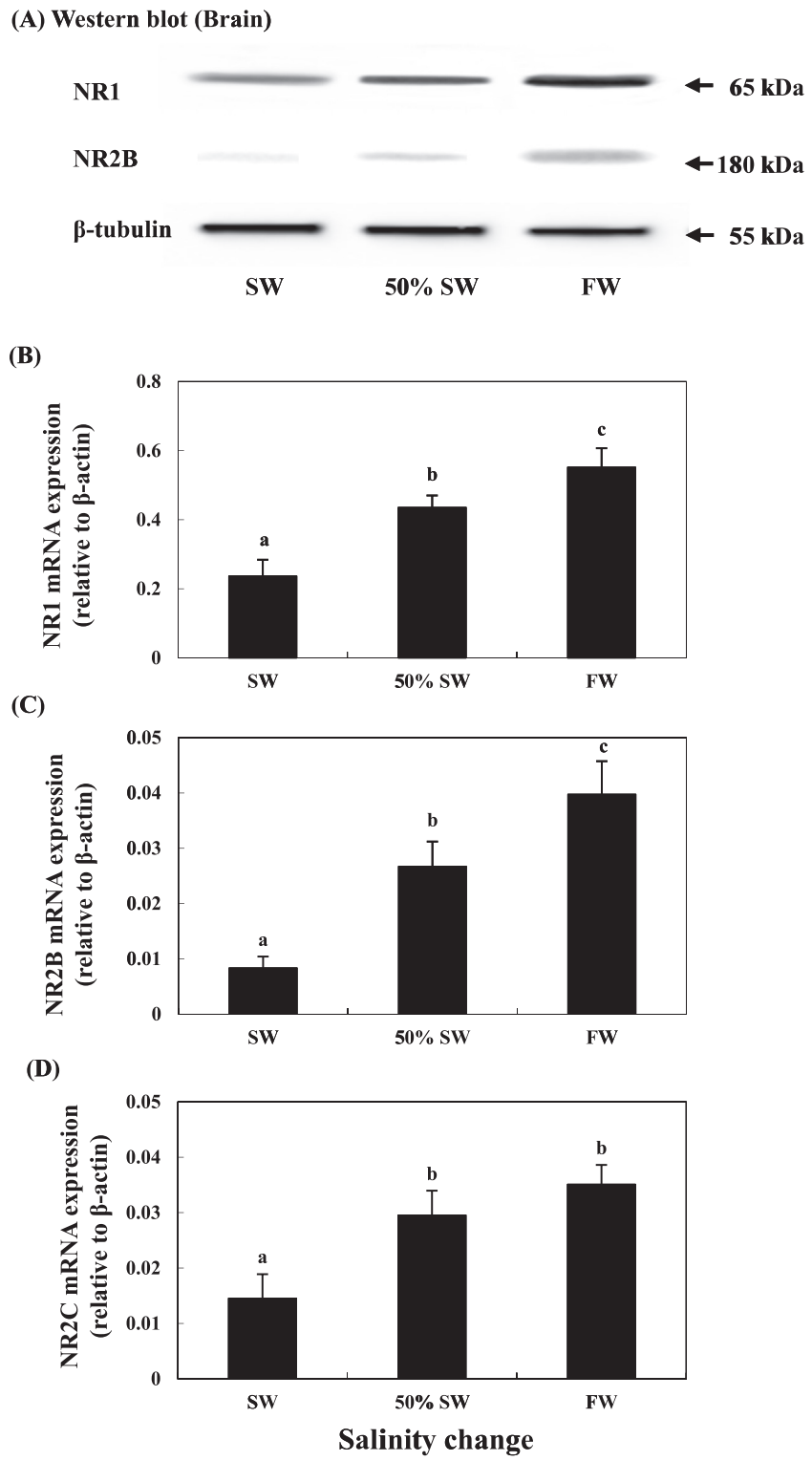

Fig. 2. Expression of N-methyl-D-aspartate receptors (NRs) mRNA and protein in the telencephalon of chum salmon after the transition from seawater (SW, 35 psu) to freshwater (FW, 0 psu). (A) Western blot of NR1 (dilution 1:4000; 65 kDa) and NR2B (dilution 1:4000; 180 kDa) protein expression in brain of chum salmon during the salinity change, with β-tubulin (55 kDa) as the internal control. Expression of NR1 (B), NR2B (C), and NR2C (D) mRNA in the telencephalon of chum salmon during salinity change using quantitative real-time PCR. We reverse transcribed 3 μg of total RNA prepared from the telencephalon and amplified the sample using gene-specific primers. Results are expressed as normalized fold expression (relative to control) with respect to β-actin levels for the same sample. Values are means ± SE ($n = 4–5$). Values with letters indicate significant differences between SW, 50% SW, and FW within the same time after salinity change ($p < 0.05$).

3.2. Expression of NR subunits mRNA and protein during salinity change

The expression of NRs mRNA in the telencephalon was highest, by a significant margin, in FW after transfer from SW (Fig. 2B, D). NR1 and NR2C were expressed in SW at levels approximately 2.3- and 2.4-fold those of the levels of expression in FW, respectively. Also, Western blot analysis detected NR1 and NR2 proteins in a size corresponding to the predicted size for chum salmon NR1 (approximately 65 kDa) and NR2 (approximately 180 kDa), and this amounts of protein were correlated with the levels of mRNA expression (Fig. 2A).

3.3. Expression of NR subunits mRNA during salinity change (in vitro)

The expression of NRs mRNA in the cultured telencephalon was highest, by a significant margin, in FW after transfer from SW (Fig. 3).

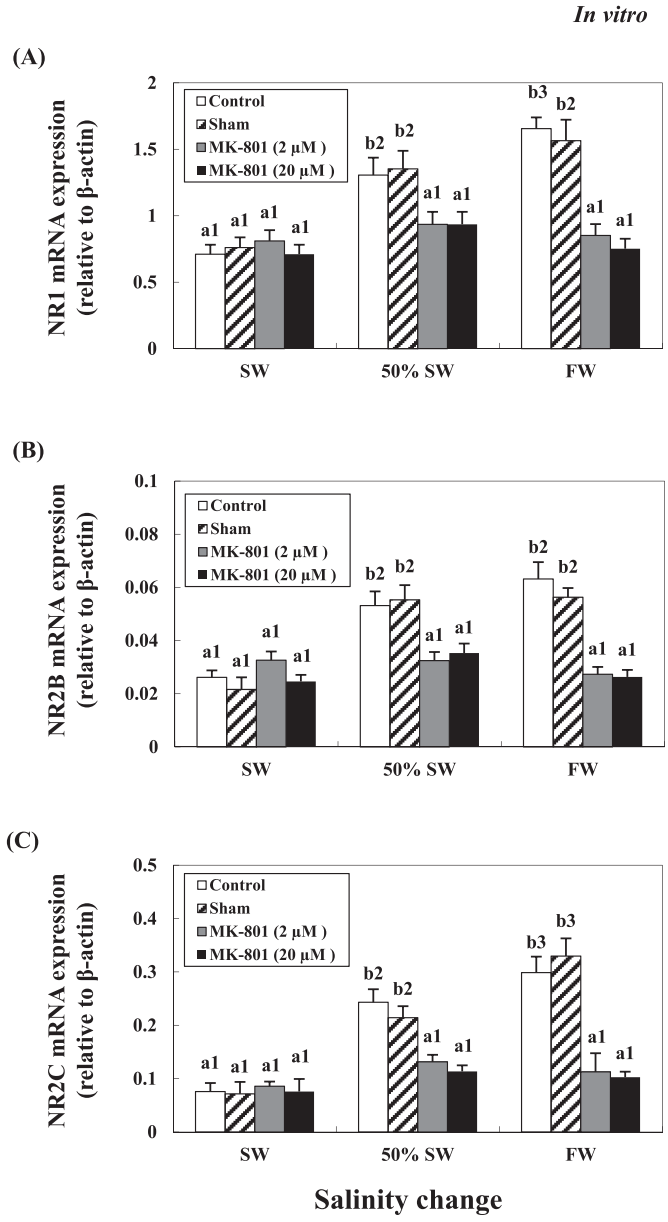

Fig. 3. Expression of N-methyl-D-aspartate receptors (NRs) (A, NR1; B, NR2B; C, NR2C) mRNA in the cultured telencephalon of chum salmon after the transition from seawater (SW, 35 psu) to freshwater (FW, 0 psu) and after MK-801 treatment. Values are means ± SE ($n = 4$–5). Values with letters indicate significant differences between SW, 50% SW, and FW within the same time after salinity change ($p < 0.05$). The numbers indicate a significant difference from the control within the same salinity and the MK-801 treatment group ($p < 0.05$).

NR1, NR2B, and NR2C were expressed in SW at levels approximately 2.3-, 2.4-, and 3.9-fold those of the levels of expression in FW, respectively. Furthermore, the expression of NR mRNA in the group that received MK-801 treatment significantly decreased, compared with the control group, after the transfer to SW.

3.4. Expression of OR mRNA and protein during salinity change

The expression of OR mRNA in the olfactory epithelium was highest, by a significant margin, in FW after transfer from SW (Fig. 4B). The level was approximately 3.5-fold that of the level in FW. Western blot analysis revealed that OR protein was detected at a size corresponding to the predicted size for chum salmon OR (approximately 45 kDa), and this amounts of protein were correlated with the levels of OR mRNA expression (Fig. 5A). The expression of OR mRNA in the cultured olfactory epithelium was highest, by a significant margin, in FW after transfer from SW (Fig. 4C). The level was approximately 21.8-fold that of the level in FW. Furthermore, the expression of OR mRNA in the group that received MK-801 treatment was significantly decreased, compared with the control group, after the transfer to FW.

3.5. Expression and activity of ACTH during salinity change

Expression of ACTH mRNAs was higher in FW than in SW, approximately 3.1-fold that of the level in FW (Fig. 5A). The plasma ACTH level was 992.7 ± 86.8 pg/mL at the start of the experiment, reached its lowest level in 50% SW of 1737.5 ± 104.0 pg/mL, and reached its lowest level of 2259.6 ± 117.4 pg/mL in FW (Fig. 5B).

3.6. Plasma cortisol and dopamine assays

Plasma cortisol levels significantly fell during the transition from SW (7.6 ± 0.4 ng/mL) to FW (3.7 ± 0.7 ng/mL) (Fig. 6). The plasma dopamine level was 564.8 ± 66.8 ng/mL at the start of the experiment and reached its lowest levels of 657.0 ± 54.0 ng/mL and 928.5 ± 77.4 ng/mL at 24 h after transfer to 50% SW and FW, respectively (Fig. 7).

4. Discussion

Changes in environmental salinity affected the expression in chum salmon of NRs, memory-associated genes; OR, olfactory imprinting-related gene; and associated mRNA, proteins, and ACTH, which affect the activity of the HPI axis.

Overall, we confirmed that salinity affected the expression of NRs (NR1, NR2B, and NR2C) and OR, which could be involved in memory storage and olfactory imprinting, respectively, in tissue-specific locations. NRs were found throughout the brain (pituitary, diencephalon, optic tectum, telencephalon, and cerebellum) and olfactory cell areas (olfactory nerve, olfactory epithelium, and olfactory bulb). NR1 and NR2C were present at very high levels in the telencephalon. Our results are similar to those for the knifefish *Apteronotus leptorhynchus*, in which Bottai et al. (1997) reported expression of NR1 observed in the telencephalon. Wenzel et al. (1995) also observed high expression levels of NR2B in the forebrain and NR2C in the telencephalon and cerebellum of rats, so our results suggest that the area of NR gene expression may be similar between fish and mammals.

Also, ORs were expressed in the telencephalon and olfactory cell areas, at particularly high levels in the olfactory epithelium. The results of this study were similar to those of previous studies in which ORs were mainly observed in the olfactory epithelia of goldfish and zebrafish (Cao et al., 1998; Saraiva and Korsching, 2007).

In the present study, we observed that salinity affected the expression of NRs and OR mRNA and proteins in the telencephalon and olfactory epithelium, respectively. The increase in NRs and OR corresponds with the degree of change from seawater to freshwater.

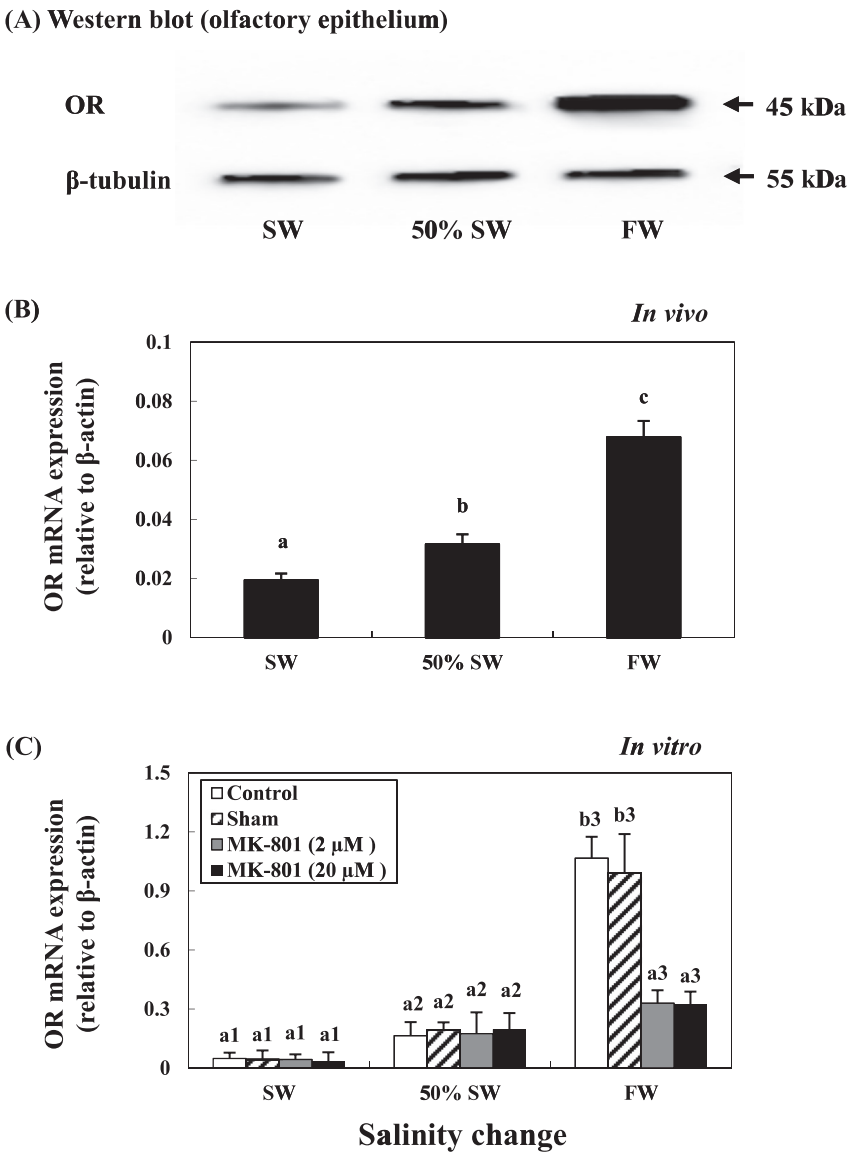

Fig. 4. Expression of olfactory receptor (OR) mRNA and protein in the olfactory epithelium and cultured olfactory epithelium of chum salmon after transition from seawater (SW, 35 psu) to freshwater (FW, 0 psu) and after MK-801 treatment. (A) Western blot of OR ($G_{\alpha\ s/olf}$ (C-18); dilution, 1:1000; 45 kDa) protein expression in olfactory epithelium of chum salmon during the salinity change, with β-tubulin (55 kDa) as the internal control. In addition, expression of ORs in olfactory epithelium (B, *in vivo*) and cultured olfactory epithelium (C, *in vitro*) of female chum salmon during salinity change was evaluated using quantitative real-time PCR. Values with letters indicate significant differences between SW, 50% SW, and FW within the same time after salinity change ($p < 0.05$). The numbers indicate a significant difference from the control within the same salinity and the MK-801 treatment group ($p < 0.05$).

The results of this study support those of a previous study by Yu et al. (2014), who reported that an increase in NR1 mRNA expression affected the learning and memory capacities of chum salmon, which were important during the transition from FW to SW. This finding suggests that NRs may play an important role in migration between the sea and the natal stream.

In addition, Fitzpatrick et al. (1986) and Hino et al. (2009) reported that olfactory system of salmonids is essential for imprinting and the subsequent homing migration as they return to their natal streams and undergo rapid salinity changes. Especially, main olfactory receptor (OR) genes detected properties of odorant substances and played olfactory imprinting and homing in salmon. Also, Kudo et al. (2009) reported that adult chum salmon have 70 times as many olfactory receptor neurons as do juveniles, so the olfactory system clearly plays a crucial role in their homing migration. Our findings agreed with those of Kudo et al. (2009), confirming that OR mRNA is expressed at higher levels in the olfactory epithelium of adults chum salmon than fry. In catadromous blue crabs *Callinectes sapidus*, which live in seawater, the number of OR cells increase if the animal transfers to fresh water, which causes enhanced sensitization of the olfactory system (Gleeson et al., 1997). Therefore, rapid salinity changes increase expression of NRs and ORs, which could be involved in memory storage and imprinting, in multiple organisms.

Compared with a control group, the mRNA expression of NRs and ORs significantly increased during salinity change from SW to FW (100% SW, 50% SW, and FW) and was highest in the FW environment (*in vitro*). However, as a result of processing the MK-801, NRs and ORs significantly decreased in both the experimental groups, but there was no distinct difference in salinity variation experiments. These results corroborated the findings of Langton et al. (2007), who reported that MK-801 accelerated the decrease in NRs, and Fiske and Brunjes (2001), who reported that MK-801 induced apoptosis of olfactory epithelial neurons, resulting in inhibition of olfactory ability. In the present study, like the previous study in chum salmon, MK-801 inhibited NR expression by acting as an antagonist of the NR, thus affecting memory. The expression of NRs in the olfactory epithelium was also

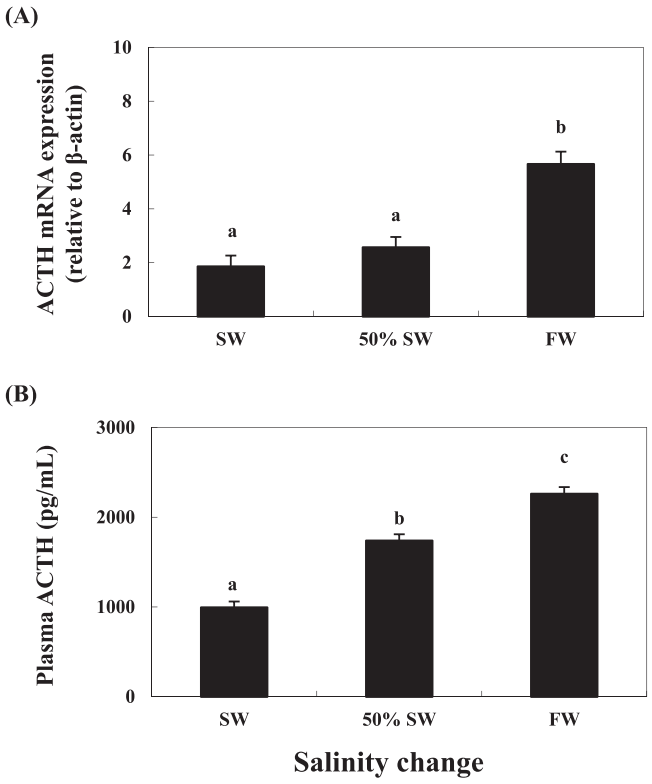

Fig. 5. Expression and activity of ACTH in chum salmon after transition from seawater (SW, 35 psu) to freshwater (FW, 0 psu). Values are means ± SE ($n = 4–5$). Values with letters indicate significant differences between SW, 50% SW, and FW within the same time after salinity change ($p < 0.05$).

inhibited by MK-801, and expression of OR was also reduced. In other words, memory capacity and olfactory imprinting ability of the salmon during their journey from the ocean to the river are closely related to the salinity of their environment.

Changing salinity was associated with important changes in the mRNA expression of HPI axis hormones, such as ACTH, in the pituitary and hypothalamus. We also examined plasma levels of ACTH and cortisol, which increased during adaptation to an FW environment. In a similar study, McCormick (2001) reported that osmoregulation of teleost adaptation was controlled by ACTH, a hormone secreted by the pituitary gland, and cortisol, a hormone secreted by the adrenal gland. Luine et al. (1994) and Nishimura et al. (1999) reported that cortisol

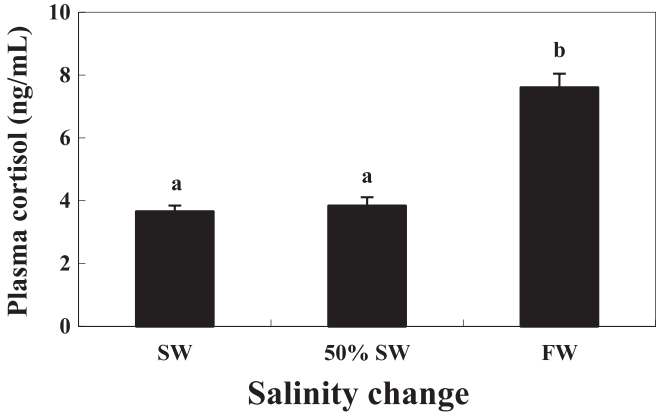

Fig. 6. Plasma cortisol levels during salinity change in female chum salmon. Values are means ± SE ($n = 4–5$). Values with letters indicate significant differences between SW, 50% SW and FW within the same time after salinity change ($p < 0.05$).

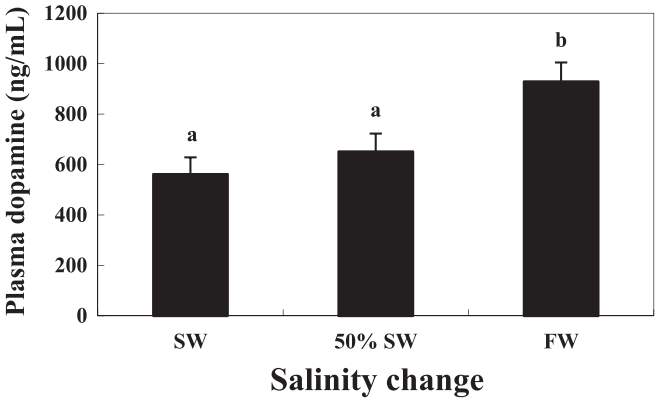

Fig. 7. Plasma dopamine levels during salinity change in female chum salmon. Values are means ± SE ($n = 4–5$). Values with letters indicate significant differences between SW, 50% SW, and FW within the same time after salinity change ($p < 0.05$).

enhances olfactory ability and has a major impact on memory capacity. Robertson and Wexler (1959) reported that adrenocortical tissue, which synthesizes cortisol, tended to be promoted excessively during the return to the natal stream. Secretion of cortisol is highest during the return to the stream for spawning, and the degree of the stress response is closely associated with the salinity change (Fagerlund, 1967; Carruth et al., 2000). In addition, Luine et al. (1994) and Cahill (1997) reported that stress reduced short-term memory capacity but enhanced long-term memory capacity. According to Dickhoff (1989), cortisol may act on OR in the brain and is directly involved on the storage and recall of olfactory imprinting and long-term memory storage. In the present study, plasma cortisol levels increased during the transfer to the FW environment, suggesting that cortisol affects the long-term memory recall associated with the storage of olfactory memories regarding the natal stream in juveniles.

Levels of dopamine in the plasma increased during adaptation to FW. Knapp et al. (1990) reported that the presence of L-glutamate increased dopamine levels. Therefore, we suggest that the dopamine increase is due to the increase in the NRs induced by salinity changes. In mammals, dopamine is involved in learning ability and formation of olfactory and long-term storage memories (Hsia et al., 1999; Pignatelli et al., 2005). Weltzien et al. (2006) also observed that in European eel *Anguilla anguilla*, levels of plasma dopamine increased during migration to SW for spawning, and that dopamine influenced the migration characteristics of eels. In the present study, salinity changes also regulated the secretion of dopamine, but dopamine levels of anadromous chum salmon followed the opposite course of those of catadromous eels, increasing during the migration upstream for spawning.

In summary, we observed that the salinity of the environment affects memory processes, imprinting, and olfactory ability in chum salmon and confirmed that cortisol and dopamine also play roles in the olfactory memories related to homing migration.

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References

Alioto, T.S., Ngai, J., 2005. The odorant receptor repertoire of teleost fish. BMC Genomics 6, 173.

Bernier, N.J., Lin, X.-W., Peter, R.E., 1999. Differential expression of corticotropin-releasing factor (CRF) and Urotensin I precursor genes, and evidence of CRF gene expression regulated by cortisol in goldfish brain. Gen. Comp. Endocrinol. 116, 461–477.

Bottai, D., Dunn, R.J., Ellis, W., Maler, L., 1997. N-methyl-D-aspartate receptor 1 mRNA distribution in the central nervous system of the weakly electric fish *Apteronotus leptorhynchus*. J. Comp. Neurol. 389, 65–80.
Buck, L.B., Axel, R., 1991. A novel multigene family may encode odorant receptors: a molecular basis for odor recognition. Cell 65, 175–187.
Cahill, L., 1997. The neurobiology of emotionally influenced memory: implications for the treatment of traumatic memory. Ann. N. Y. Acad. Sci. 821, 238–246.
Cao, Y., Oh, B.C., Stryer, L., 1998. Cloning and localization of two multigene receptor families in goldfish olfactory epithelium. Proc. Natl. Acad. Sci. U. S. A. 95, 11987–11992.
Carruth, L.L., Dores, R.M., Maldonado, T.A., Norris, D.O., Ruth, T., Jones, R.E., 2000. Elevation of plasma cortisol during the spawning migration of a landlocked kokanee salmon (*Oncorhynchus nerka kennerlyi*). Comp. Biochem. Physiol. C 127, 123–131.
Choi, Y.J., Kim, N.N., Shin, H.S., Choi, C.Y., 2014. The expression of leptin, estrogen receptors, and vitellogenin mRNAs in migrating female chum salmon, *Oncorhynchus keta*: the effects of hypo-osmotic environmental changes. Asian-Aust. J. Anim. Sci. 27, 479–487.
Cox, J.A., Kucenas, S., Voigt, M.M., 2005. Molecular characterization and embryonic expression of the family of N-methyl-D-aspartate receptor subunit genes in the zebrafish. Dev. Dyn. 234, 756–766.
Davila, N.G., Blakemore, L.J., Trombley, P.Q., 2003. Dopamine modulates synaptic transmission between rat olfactory bulb neurons in culture. J. Neurophysiol. 90, 395–404.
Dickhoff, W.W., 1989. Salmonids and annual fishes: Death after sex. In: Schreibman, M.P., Scanes, C.G. (Eds.), Development, Maturation, and Senescence of Neuroendocrine Systems: A Comparative Approach. Academic Press, New York, pp. 253–268.
Dittman, A.H., Quinn, T.P., 1996. Homing in pacific salmon: mechanisms and ecological basis. J. Exp. Biol. 19983–19991.
Doyon, C., Leclair, J., Trudeau, V.L., Moon, T.W., 2006. Corticotropin-releasing factor and neuropeptide Y mRNA levels are modified by glucocorticoids in rainbow trout, *Oncorhynchus mykiss*. Gen. Comp. Endocrinol. 146, 126–135.
Fagerlund, U.H.M., 1967. Plasma cortisol concentration in relation to stress in adult sockeye salmon during the freshwater stage of their life cycle. Gen. Comp. Endocrinol. 8, 197–207.
Fiske, B.K., Brunjes, P.C., 2001. NMDA receptor regulation of cell death in the rat olfactory bulb. J. Neurobiol. 47, 223–232.
Fitzpatrick, M.S., Van Der Kraak, G., Schreck, C.B., 1986. Plasma profiles of sex steroids and gonadotropin in coho salmon (*Oncorhnychus kisutch*) during final maturation. Gen. Comp. Endocrinol. 62, 437–451.
Fukaya, M., 1999. Molecular Histochemical and Pharmaco-behavioral Studies on the Glutamate Receptor in the Brain of Salmonid Fishes. (Master Thesis). Hokkaido University, Hakodate.
Gleeson, R.A., Wheatly, M.G., Reiber, C.L., 1997. Perireceptor mechanisms sustaining olfaction at low salinities: insight from the euryhaline blue crab, *Callinectes sapidus*. J. Exp. Biol. 200, 445–456.
Groot, C., Margolis, L., 1991. Pacific salmon life histories, published in co-operation with the government of Canada. Dep. Fish. Ocean 9–11.
Hara, T.J., 1994. Olfaction and gustation in fish: an overview. Acta Physiol. Scand. 152, 207–217.
Hasler, A.D., Scholz, A.T., 1983. Olfactory Imprinting and Homing in Salmon: Investigations Into the Mechanism of the Imprinting Process. Springer-Verlag, Berlin.
Hino, H., Miles, N.G., Bandoh, H., Ueda, H., 2009. Molecular biological research on olfactory chemoreception in fishes. J. Fish Biol. 75, 945–959.
Hsia, A.Y., Vincent, J.D., Lledo, P.M., 1999. Dopamine depresses synaptic inputs into the olfactory bulb. J. Neurophysiol. 82, 1082–1085.
Huising, M.O., Metz, J.R., van Schooten, C., Taverne-Thiele, A.J., Hermsen, T., Verburg-van Kemenade, B.M., Flik, G., 2004. Structural characterization of a cyprinid (*Cyprinus carpio* L.) CRH, CRHBP and CRH-R1, and the role of these proteins in the acute stress response. J. Mol. Endocrinol. 32, 627–648.
Jeon, C.H., Kim, S.R., Kim, W.S., Lee, C.H., Seong, K.B., Lee, C.S., Oh, M.J., Kim, J.H., 2011. Monitoring of viruses in chum salmon (*Oncorhynchus keta*) migrating to Korea. Arch. Virol. 156, 1025–1030.
Johnson, M.A., Banks, M.A., 2011. Sequence conservation among orthologous vomeronasal type 1 receptor-like (ora) genes does not support the differential tuning hypothesis in Salmonidae. Gene 485, 16–21.
Johnstone, K.A., Lubieniecki, K.P., Koop, B.F., Davidson, W.S., 2012. Identification of olfactory receptor genes in Atlantic salmon *Salmo salar*. J. Fish Biol. 81, 559–575.
Kinoshita, M., Hosokawa, T., Urano, A., Ito, E., 2004. Long-term potentiation in the optic tectum of rainbow trout. Neurosci. Lett. 370, 146–150.
Kinoshita, M., Fukaya, M., Tojima, T., Kojima, S., Ando, H., Watanabe, M., Urano, A., Ito, E., 2005. Retinotectal transmission in the optic tectum of rainbow trout. J. Comp. Neurol. 484, 249–259.
Knapp, A.G., Schidt, K.F., Dowling, J.E., 1990. Dopamine modulates the kinetics of ion channels gated by excitatory amino acids in retinal horizontal cells (kainate/catecholamine/cei culture/patch clamp/noise analysis). Proc. Natl. Acad. Sci. U. S. A. 87, 767–771.
Kudo, H., Shinto, M., Sakurai, Y., Kaeriyama, M., 2009. Morphometry of olfactory lamellae and olfactory receptor neurons during the life history of chum salmon (*Oncorhynchus keta*). Chem. Senses 34, 617–624.
Langton, J.M., Kim, J.H., Nicholas, J., Richardson, R., 2007. The effect of the NMDA receptor antagonist MK-801 on the acquisition and extinction of learned fear in the developing rat. Learn. Mem. 14, 665–668.
Luine, V., Villegas, M., Martinez, C., McEwen, B.S., 1994. Repeated stress causes reversible impairments of spatial memory performance. Brain Res. 639, 167–170.
Machidori, S., Katou, F., 1984. Spawning populations and marine life of masu salmon (*Oncorhynchus masou*). Int. North Pac. Fish. Comm. 43, 1–138.
McCormick, S.D., 2001. Endocrine control of osmoregulation in teleost fish. Am. Zool. 41, 781–794.
Mombaerts, P., 2004. Genes and ligands for odorant, vomeronasal and taste receptors. Nat. Rev. Neurosci. 5, 263–278.
Mommsen, T.P., Vijayan, M.M., Moon, T.W., 1999. Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. Rev. Fish Biol. Fish. 9, 211–268.
Nishimura, J., Endo, Y., Kimura, F., 1999. A long-term stress exposure impairs maze learning performance in rats. Neurosci. Lett. 273, 125–128.
Péqueux, A., 1995. Osmotic regulation in crustaceans. J. Crustac. Biol. 15, 1–60.
Pignatelli, A., Kobayashi, K., Okano, H., Belluzzi, O., 2005. Functional properties of dopaminergic neurons in the mouse olfactory bulb. J. Physiol. 564, 501–514.
Putman, N.F., Jenkins, E.S., Michielsens, C.G.J., Noakes, D.L.G., 2014. Geomagnetic imprinting predicts spatiotemporal variation in homing migration of pink and sockeye salmon. J. R. Soc. Interface 11, 20140542.
Quinn, R.P., 2005. The Behavior and Ecology of Pacific Salmon and Trout. University of Washington Press, Seattle (WA).
Robertson, O.H., Wexler, B.C., 1959. Hyperplasia of the adrenal cortical tissue in Pacific salmon (*genus Oncorhynchus*) and rainbow trout (*Salmo gairdnerii*) accompanying sexual maturation and spawning. Endocrinology 65, 225–238.
Salo, E.O., 1991. Life history of chum salmon (*Oncorhynchus keta*). In: Groot, C., Margolis, L. (Eds.), Pacific Salmon Life Histories. Columbia Press, Vancouver, pp. 231–309.
Saraiva, L.R., Korsching, S.I., 2007. A novel olfactory receptor gene family in teleost fish. Genome Res. 17, 1448–1457.
Sison, M., Gerlai, R., 2011. Behavioral performance altering effects of MK-801 in zebrafish (*Danio rerio*). Behav. Brain Res. 220, 331–337.
Sorensen, P.W., Scott, A.P., Stacey, N.E., Bowdin, L., 1995. Sulfated 17, 20 beta-dihydroxy-4-pregnen-3-one functions as a potent and specific olfactory stimulant with pheromonal actions in the goldfish. Gen. Comp. Endocrinol. 100, 128–142.
Tipsmark, C.K., Sørensen, K.J., Madsen, S.S., 2010. Aquaporin expression dynamics in osmoregulatory tissues of Atlantic salmon during smoltification and seawater acclimation. J. Exp. Biol. 213, 368–379.
Tzeng, D.W., Lin, M.H., Chen, B.Y., Chen, Y.C., Chang, Y.C., Chow, W.Y., 2007. Molecular and functional studies of tilapia (*Oreochromis mossambicus*) NMDA receptor NR1 subunits. Comp. Biochem. Physiol. 146, 402–411.
Ueda, H., 2011. Physiological mechanism of homing migration in Pacific salmon from behavioral to molecular biological approaches. Gen. Comp. Endocrinol. 170, 222–232.
Ueda, H., Kaeriyama, M., Urano, A., Kurihara, K., Yamauchi, K., 1995. Homing mechanisms in salmon: roles of vision and olfaction. In: Goetz, F.W., Thomas, P. (Eds.), Proceedings of the Fifth International Symposium on the Reproductive Physiology of Fish. Fish Symposium 95. Austin, Texas, pp. 218–220 (Austin, Texas).
Weltzien, F.-A., Pasqualini, C., Sébert, M.E., Vidal, B., Le Belle, N., Kah, O., Vernier, P., Dufour, S., 2006. Androgen-dependent stimulation of brain dopaminergic systems in the female European eel (*Anguilla anguilla*). Endocrinology 147, 2964–2973.
Wenzel, A., Scheurer, L., Künzi, R., Fritschy, J.M., Mohler, H., Benke, D., 1995. Distribution of NMDA receptor subunit proteins NR2A, 2B, 2C and 2D in rat brain. Neuroreport 7, 45–48.
Xia, S., Miyashita, T., Fu, T.F., Lin, W.Y., Wu, C.L., Pyzocha, L., Lin, I.R., Saitoe, M., Tully, T., Chiang, A.S., 2005. NMDA receptors mediate olfactory learning and memory in *Drosophila*. Curr. Biol. 15, 603–615.
Yu, J.-N., Ham, S.H., Lee, S.I., Jin, H.-J., Ueda, H., Jin, D.-H., 2014. Cloning and characterization of the N-methyl-D-aspartate receptor subunit NR1 gene from chum salmon, *Oncorhynchusketa* (Walbaum, 1792). SpringerPlus 3, 9.