Changes of physiological rhythms of N-methyl-d-aspartate receptors in the chum salmon Oncorhynchus keta: effect of seawater acclimation during the parr-smolt transformation

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Changes of physiological rhythms of *N*-methyl-D-aspartate receptors in the chum salmon *Oncorhynchus keta*: effect of seawater acclimation during the parr-smolt transformation

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We examined changes on *N*-methyl-D-aspartate receptors (NRs) in different growth stages (early parr, parr, and early smolt) of chum salmon, *Oncorhynchus keta,* during parr-smolt transformation from freshwater to seawater. Expression levels of NR genes mRNA and concentration of cortisol, T_3, T_4, dopamine and Na^+/K^+-ATPase activity significantly increased at salinity change condition. Moreover, in cultured brain cells, NRs were significantly lower in all groups treated with MK-801 (an antagonist of NRs) than in the early parr stage group in the FW treatment. We confirmed that the reduction in mRNA expression levels of NRs increased from the early parr to the early smolt stage. The information reported here should be taken into account in future studies on the relationship between memory factors of natal streams and homing mechanisms in Salmonidae.

Keywords: chum salmon; *N*-methyl-D-aspartate receptors; NR antagonist; parr-smolt transformation; physiological rhythm; seawater acclimation

1. Introduction

Olfaction of fish is an important sensory system, essential for processing biologically relevant information throughout the life history, and also it plays a role in the detection of pheromones associated with breeding, kin recognition, conspecific attraction, and predator avoidance (Johnson et al. 2006; Rafferty & Boughman 2006). Furthermore, the olfactory sense often controls homing behavior for many species through detection of home site-specific cues. For instance, black rockfish *Sebastes inermis* when moved only tens of meters away from their home territory can rely on olfaction to return to their home habitat, which demonstrates homing is important, even on a small scale (Mitamura et al. 2005). In salmonids, such as the Atlantic salmon *Salmo salar* and the Pacific salmon *Oncorhynchus* spp., olfaction is essential for successful completion of the homing migration (Hasler & Scholz 1983; Johnstone et al. 2012).

Salmonids migrate to the ocean after undergoing complex morphological, physiological, and behavioral changes, known as the parr-smolt transformation (PST), smoltification, or smolting (McCormick & Saunders 1987). Hasler and Scholz (1983) suggested that the PST is a critical learning period for imprinting. After smolting, juveniles migrate to the sea where they feed for one-to-four years, acquiring >99% of their

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adult weight. When they are ready to spawn, salmon may travel 2000–4000 km from the ocean feeding grounds to their natal freshwater (FW) (river) streams to spawn (Quinn 2005). Stabell (1992) and Carruth et al. (2002) suggested that streams must have a unique chemical composition, which persists year after year, and that salmon are able to discriminate between their natal stream and other streams.

Olfactory memory studies have recently focused on the possible role of *N*-methyl-D-aspartate receptors (NRs) on the formation of memory (Kinoshita et al. 2004, 2005; Xia et al. 2005). The NRs are ligand-gated ionotropic glutamate receptors that are important mediators for neuronal events, such as synaptic plasticity, learning and memory, neuronal development, and circuit formation (Yu et al. 2014). The study of NRs has focused on long-term memory, which is mainly responsible for memory control in the brain (Kinoshita et al. 2004, 2005). NRs regulate the immediate-early gene expression, improving the long-term storage capacity of the brain, and cause an increase in expression of genes associated with olfactory imprinting during PST (Fukaya 1999; Kinoshita et al. 2004, 2005).

NRs are heteromers comprising NR subunits, NR1 and NR2, coding one NR1 and four NR2 (NR2A-D) (Yu et al. 2014). MK-801, an antagonist of NR, has been used to understand the function of NRs in terms of memory performance and learning ability in fish and mammals (Carey et al. 1998; Takahashi et al. 2010). Sison and Gerlai (2011) reported that MK-801 reduced swimming speed and brain perception of zebrafish, confirming the importance of NRs in fish.

Olfactory memories of salmon are engraved during PST. During this transformation, hormones, such as thyroxine (T_4), cortisol, insulin-like growth factor-1, growth hormone, and dopamine, a neurotransmitter increase in the plasma. Kudo et al. (1994) and Morin et al. (1995) reported that T_4, triiodothyronine (T_3), and dopamine, which are involved in the storage of olfactory memories, continuously increased during PST.

Cortisol is often referred to as a seawater (SW) adapting hormone because it is involved in the ability of fish to maintain water and electrolyte balance when they are in SW environments (Mommsen et al. 1999). Moreover, increased cortisol levels contribute to the regulation of PST. Veillette et al. (1995) reported that cortisol levels regulate water absorption in the intestines of Atlantic salmon *S. salar* during PST.

During changes in salinity, fish adjust plasma osmolality by detecting changes in Na^+ and Cl^- ions as water flows across specialized osmoregulatory organs, such as the gills (Evans 1993). Fish generally maintain a constant body homeostasis, despite changes in osmolality caused by environmental salinity change. For instance, one of the most important enzymes associated with ion regulation in the gills of fish is Na^+/K^+-ATPase (NKA) because it energizes the branchial excretion of Na^+ and Cl^- from marine teleosts (McCormick 1995; Marshall & Bryson 1998; Evans et al. 1999). NKA is important for maintaining homeostasis and for regulating many transport systems in a variety of fish osmoregulatory organs, including the gills (McCormick 1995). In addition, McCormick and Saunders (1987) reported that NKA in the gill epithelium, which are involved in osmoregulation, increased during PST.

The present study investigated the memory and imprinting ability of chum salmon *Oncorhynchus keta* fry during PST, in response to salinity changes when discharged into the sea. We divided the PST period into early parr, parr, and early smolt stages, and recorded, therefore, the response of 2-, 3-, and 4-month-old chum salmon to sequential salinity changes [FW, 25% SW, 50% SW, 75% SW, and SW]. We analyzed changes in mRNA and protein expression of NRs (NR1, NR2B, and NR2C) during PST, and confirmed the effect of MK-801 on NRs and on the growth process during this period

(PST). In addition, we have determined the effects of NRs on the imprinted physiological response and olfactory memory by measuring concentration of plasma cortisol, T_4, T_3, dopamine, and gills NKA activity.

2. Materials and methods

2.1. Experimental fish

Chum salmon *O. keta* were obtained from the Gyeongsangnam-do Fisheries Resources Research Institute (Gyeongsangnam-do, Korea) in February 2014. Fish were reared in eight 200-L circulation filter tanks for 2 months, prior to the beginning of the experiments, at the Marine Molecular & Environmental Physiology Lab, Korea Maritime and Ocean University, Korea.

Fish were selected according to Hutchison and Iwata (1997) and divided into the different stages of PST: early parr (appearing of parr marks, 2.2 ± 0.3 cm, 0.6 ± 0.2 g), parr (clean parr marks, 5.4 ± 0.4 cm, 1.5 ± 0.2 g), and early smolt (no parr marks, 11.4 ± 0.5 cm, 3.5 g).

Additionally, a specific protocol was used to transfer chum salmon from FW (0 psu) to SW (35 psu). Briefly, groundwater was poured into square 100-L tanks, and fish were subsequently kept at 25% SW, 50% SW, and 75% SW for 24 h periods by adding SW until all tanks had only SW. Temperature was maintained at 12 ± 0.5 °C, and photoperiod was maintained at a 12:12 h light–dark cycle.

2.2. Sampling

Ten fish from each group (FW, 25% SW, 50% SW, 75% SW, and SW) were randomly selected for blood and tissue sampling at 11:00. Immediately after collection, tissue samples were frozen in liquid nitrogen and stored at −80 °C until total RNA extraction was performed.

In addition, a blood sample was collected from the caudal vasculature using a 1 mL syringe coated with heparin. After centrifugation (4 °C, 10,000×*g*, 5 min), the plasma was stored at −80 °C until analysis. Specially, the amount of blood was very low for the analysis from a single chum salmon fry. So blood was taken from 15 chum salmon fry in each aquarium and mixed during analysis.

2.3. Brain cell culture

Each brain region cultured using enzymatic and mechanical procedures and rapidly removed and placed in 3 mL ice-cold dispersion buffer (Dulbecco's phosphate-buffered saline, without calcium chloride and magnesium chloride, containing 100 U/mL penicillin, 100 μg/mL streptomycin, and 2.5 μg/mL fungizone; GIBCOBRL, Rockville, MD). The isolated brain tissues were then transferred to 6 mL of fresh dispersion buffer containing 0.25% trypsin (Type II-S from porcine pancreas; Sigma, St. Louis, MO). The mixture of dispersed hypothalamus cells and tissues was filtered, and the culture medium (Gibco-BRL) was added. Cultured brain cells were sampled at 24 h intervals during the transition of fish from FW to SW; each sample was centrifuged (20 °C, 10,000×*g*, 15 s), and the supernatant was discarded and stored at −80 °C until further RNA extraction.

MK-801 (M107; Sigma, St. Louis, MO, USA), was dissolved in 0.8% physiological saline and added to the culture medium [concentrations 2 and 20 μM MK-801]. Each

sample was centrifuged (20 °C, 10,000×*g*, 15 s), and the supernatant was discarded and stored at −80 °C until RNA extraction.

2.4.   Tissue distribution of NR mRNAs

To examine the mRNA expression levels of NR subunits (NR1, NR2B, and NR2C) in tissues, total RNA were extracted from the pituitary, diencephalon, optic tectum, telencephalon, cerebellum, olfactory nerve, olfactory epithelium, and olfactory bulb. Total RNA was extracted using Tri-Reagent (MRC, Cincinnati, OH, USA), and reverse transcription was performed using M-MLV reverse transcriptase (Promega, OH, USA). PCR amplification was performed with specific primer sets (Table 1) with a Ex Taq (RR001A, TaKaRa, Tokyo, Japan). 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 (Table 1). 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 PCR (QPCR)

QPCR was conducted to determine the relative mRNA expression of NRs using the total RNA extracted. Primers for QPCR were designed with reference to the known sequences of chum salmon (GenBank accession nos.: NR1, *JQ924060*; NR2B, *KM509062*; NR2C, *KF595125*) (Table 1). The PCR amplification was conducted using a Bio-Rad CFX96™ Real-time PCR Detection System (Bio-Rad, USA) and iQ™ SYBR Green Supermix (Bio-Rad, USA) according to the manufacturer's instructions. The QPCR was performed as follows: 95 °C for 5 min, followed by 50 cycles each of 95 °C for 20 s and 55 °C for 20 s. As internal controls, experiments were duplicated with β-actin, and all data were expressed relative to the corresponding β-actin calculated threshold cycle (ΔCt) levels. The calibrated ΔCt value (ΔΔCt) for each sample and internal controls (β-actin) was calculated using the $2^{-\Delta\Delta Ct}$ method [ΔΔCt = $2^{\wedge} - (\Delta Ct_{sample} - \Delta Ct_{internal\ control})$].

2.6.   Western blot analysis

Total protein isolated from the brain was extracted using a T-PER® Tissue Protein Extraction Reagent (Thermo fisher scientific, Inc., USA) according to the manufacturer's instructions, and quantified using the Bradford method (Bio-Rad). A total of 30 μg of

Table 1.   Primers used for amplification of PCR.

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′
β-actin	Forward	5′-ATC TGG CAT CAC ACC TTC TA-3′
	Reverse	5′-CTT CTC CCT GTT GGC TTT G-3′

total protein was loaded per lane onto Mini-PROTEAN® TGX™ Gels (Bio-Rad, USA). Samples were electrophoresed at 180 V. The gels were immediately transferred to a 0.2 μm polyvinylidene difluoride membrane (Bio-Rad, USA) at 85 V for 3 min using the Trans-Blot® Turbo™ Transfer System. Membranes were blocked with 5% milk in Tris-buffered saline (TBS; pH 7.4) for 45 min and washed in TBS. Membranes were incubated with polyclonal rabbit anti-NR1 (dilution, 1:4000; NR1D2, ARP38568; Aviva System Biology, CA, USA) and anti-NR2 (dilution, 1:4000; NR2B, AB1557P; Millipore, CA, USA) followed by incubation with anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase (dilution 1:4000; Bio-Rad, Hercules, CA, USA) for 60 min. The internal control was β-tubulin (dilution 1:2000; ab6046, Abcam, Cambridge, UK), followed by horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (1:4000; Bio-Rad, Hercules, CA, USA) for 60 min. Immunoreactive bands were detected using WesternBright™ ECL (Advansta, Menlo Park, CA, USA) with a 30 s exposure, using a Molecular Imager® ChemiDoc™ XRS$^+$ System (Bio-Rad, Hercules, CA, USA). 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 ratio of internal control (β-tubulin)/NRs was calculated for each concentration and plotted against the concentration of the internal control.

2.7. NKA activity

NKA activity was measured according to the methods described by Uchida et al. (1996) with modifications. Gill (approximately 4–6 primary gill filaments from just above the septum) were collected from the anesthetized fish, immersed in 100 μL of ice-cold SEI buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole, pH 7.3), and frozen at −80 °C. The filaments were thawed, homogenized in SEI buffer containing 0.1% deoxycholic acid and centrifuged at $5000 \times g$ for 30 s to remove insoluble materials. The supernatant was assayed for NKA activity and protein content. Homogenate samples (10 μL) were added to a 200-μL assay mixture with and without 0.5 mmol/L ouabain in 96-well microplates at 25 °C and read at 340 nm for 10 min with intermittent mixing. Protein content of the sample was determined by Protein Assay Standard II (Bio-Rad, USA). The NKA activity was calculated as the difference in ATP hydrolysis between the presence and absence of ouabain, and expressed as l mol of ADP per mg protein per hour.

2.8. Plasma parameter analysis

The levels of cortisol, T_3, T_4, and dopamine in the plasma were analyzed using enzyme immunoassay (ELISA) kits for fish (Cusabio Biotech ELISA kit catalog no.; cortisol, E12121Fh; T_3, E08488f; T_4, E08489f; dopamine, EQ027496FI; Cusabio Biotech, Hubei, China), according to the manufacturer's instructions. The optical density of each well was determined using a microplate reader set to 450 nm.

2.9. Statistical analysis

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

3. Results

3.1. *Tissue distribution of NR mRNAs*

Figure 1 shows the tissue-specific mRNA expression patterns of chum salmon NR subunits (NR1, NR2B, and NR2C) in FW. NR mRNA was detected in all brain and olfactory tissues, especially in the diencephalon and telencephalon.

3.2. *Expression of NRs mRNA (*in vivo*)*

The mRNA expression levels of NRs in the telencephalon of chum salmon increased from FW to SW [significantly increased in early smolts, approximately 1.16- (NR1; Figure 2(b)), 1.03- (NR2B; Figure 2(c)), and 2.07-fold (NR2C; Figure 2(d)) higher] (Figure 2). Moreover, NR mRNA expression in the early smolt stage was significantly higher than in the early parr stage [approximately 1.54- (NR1; Figure 2(b)), 1.33- (NR2B; Figure 2(c)), and 1.51-fold (NR2C; Figure 2(d)) higher].

Western blot analysis revealed a protein containing the NR1 and NR2B, with a mass similar to the expected mass for chum salmon NR1 (65 kDa) and NR2B (180 kDa). The mRNA expression pattern of this protein resembled that of the NR1 and NR2 in chum salmon.

3.3. *Expression of NRs mRNA (*in vitro*)*

The mRNA expression levels of NRs were observed in the cultured brain of chum salmon during salinity changes and exposure to MK-801 [Figures 3 (early parr), 4 (parr), and 5 (early smolt stage group)]. In the cultured brain of early parr stage salmon, NRs mRNA expression levels significantly increased with salinity; however, NR2B and NR2C mRNA expression levels peaked at 50% SW, and then decreased (Figure 3). In parr stage salmon, NR2C mRNAs peaked at 75% SW, and decreased at SW salinity (Figure 4(c)). In early smolt stage salmon, NR1 and NR2C mRNAs significantly
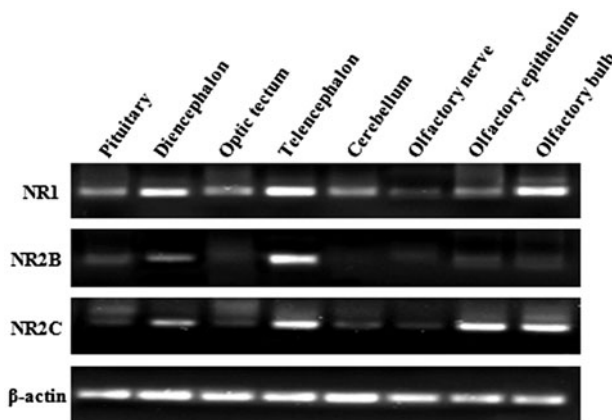

Figure 1. Distribution of chum salmon NRs in tissues (pituitary, diencephalon, optic tectum, telencephalon, cerebellum, olfactory nerve, olfactory epithelium, and olfactory bulb).
Note: RT-PCR analysis of NRs transcripts in different tissues collected in February 2014 shown in a 1.0% agarose electrophoresis gel with ethidium bromide staining.
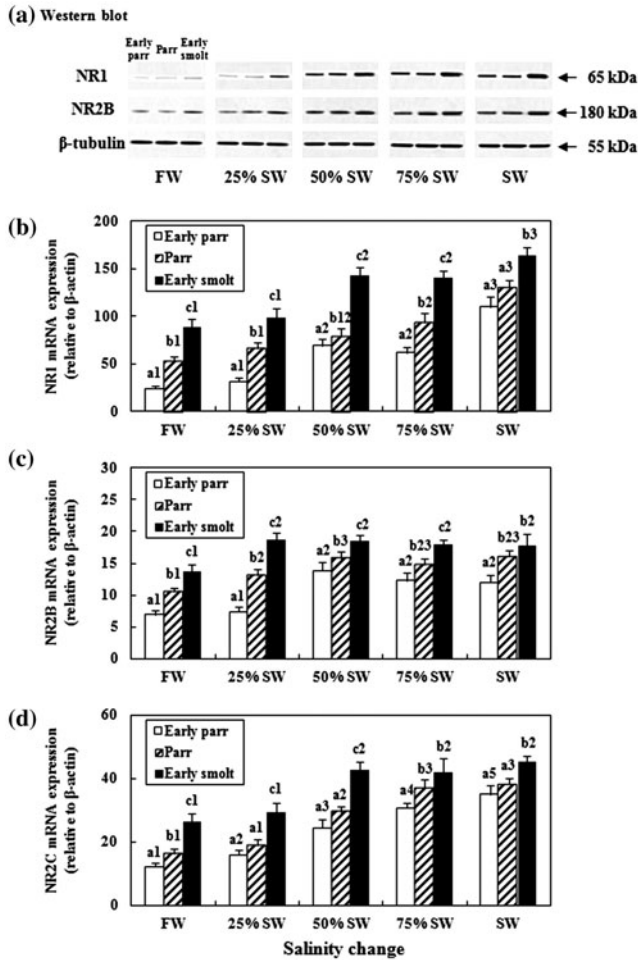
Figure 2.   Expression of NR proteins (a) and mRNAs ((b), NR1; (c), NR2B; (d), NR2C) in the telencephalon of early parr, parr, and early smolt stages of chum salmon (2, 3, and 4 months after hatching) transferred from freshwater (FW, 0 psu) to seawater (SW, 35 psu) using quantitative real-time PCR.
Notes: Results are expressed as normalized fold expression (relative to control) with respect to β-actin levels for the same sample, and values are means ± SE (n = 15). Letters indicate significant differences between PST period groups within the same salinity concentration. Numbers indicate a significant difference between salinity concentrations within the same PST period group ($p < 0.05$).

increased up to 50% SW, and stabilized from then on (Figure 5(a) and (c)). In addition, the expression of NR mRNAs in the MK-801 treatment group significantly decreased after fish were transferred to SW when compared to the control group.

3.4. *Levels of plasma cortisol and gill NKA activity*

The levels of cortisol and gill NKA activity are shown in Figure 6. Cortisol (Figure 6(a); early parr, 11.0 ± 2.3; parr, 16.3 ± 2.6; and early smolt, 36.8 ± 2.7 ng/mL) and gill NKA
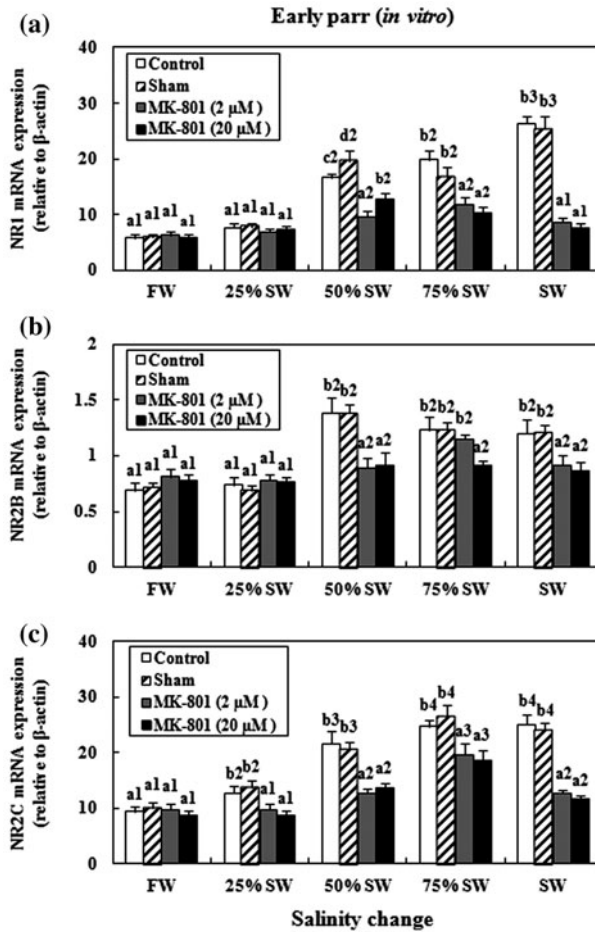
Figure 3.   mRNA expression levels of NRs in cultured brain cells of early parr chum salmon (2 months after hatching) transferred from freshwater (FW, 0 psu) to seawater (SW, 35 psu) and after MK-801 treatment using quantitative real-time PCR.
Notes: Values are means ± SE ($n = 15$). Letters indicate significant differences between MK-801 treatment groups within the same salinity concentration. Numbers indicate a significant difference between salinity concentrations within the MK-801 treatment group ($p < 0.05$).

(Figure 6(c); early parr, 21.0 ± 1.1; parr, 25.2 ± 1.4; and early smolt, 29.8 ± 1.2 µmol/ADP mg protein/h) levels in the early smolt stage significantly increased from FW to SW (cortisol, 81.4 ± 3.4 ng/mL; gill NKA, 63.2 ± 1.6 µmol/ADP mg protein/h).

3.5.   *Levels of T_3, T_4, and dopamine in the plasma*

The levels of T_3, T_4, and dopamine in the plasma are shown in Figure 7. T_3 (Figure 7(a); early parr, 182.7 ± 11.1; parr, 252.7 ± 15.5; and early smolt, 300.9 ± 22.7 ng/mL), T_4 (Figure 7(b); early parr, 2.3 ± 0.1; parr, 2.7 ± 0.2; and early smolt, 3.9 ± 0.4 ng/mL), and dopamine (Figure 7(c); early parr, 234.1 ± 22.5; parr, 364.5 ± 33.5; and early smolt, 410.8 ± 30.7 ng/mL) levels in the early smolt stage significantly
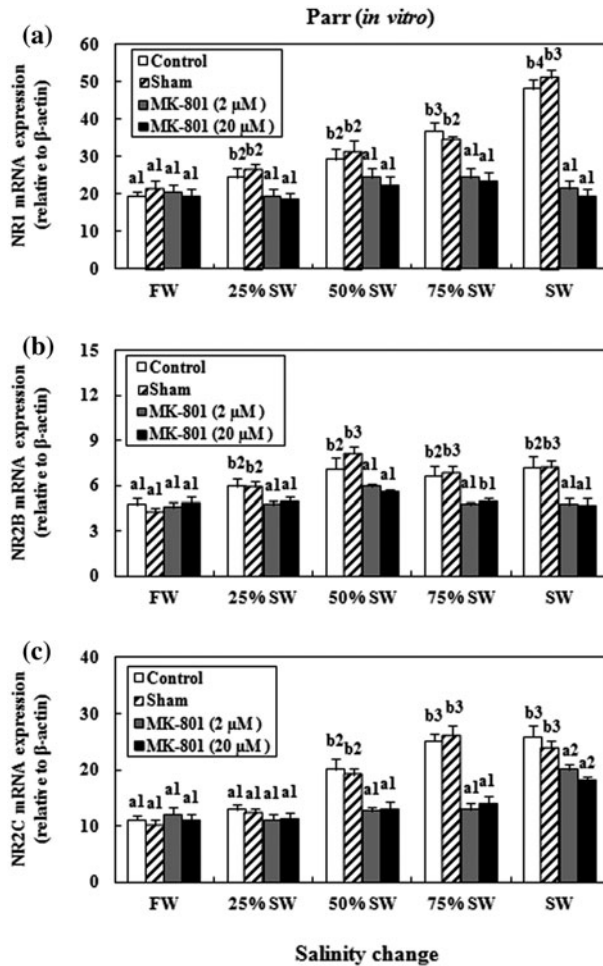
Figure 4. mRNA expression levels of NRs in cultured brain cells of parr chum salmon (3 months after hatching) transferred from freshwater (FW, 0 psu) to seawater (SW, 35 psu) and after MK-801 treatment using quantitative real-time PCR.

Notes: Values are means ± SE ($n = 15$). Letters indicate significant differences between MK-801 treatment groups within the same salinity concentration. Numbers indicate a significant difference between salinity concentrations within the MK-801 treatment group ($p < 0.05$).

increased from FW to SW (T_3, 630.2 ± 53.5 ng/mL; T_4, 7.7 ± 0.6 ng/mL and dopamine, 870.2 ± 55.8 ng/mL).

4. Discussion

In this study, we investigated the salinity-adaptive capacity, memory, and imprinting ability of different salmon fry growth stages (early parr, parr, and early smolt stages) as a response to salinity changes when discharged into the sea.

Overall, NR1, NR2B, and NR2C were expressed in the main brain tissues involved in olfactory memory. NR1 and NR2C were very high in the diencephalon, telencephalon, and olfactory bulb, while NR2B was especially high in the telencephalon.
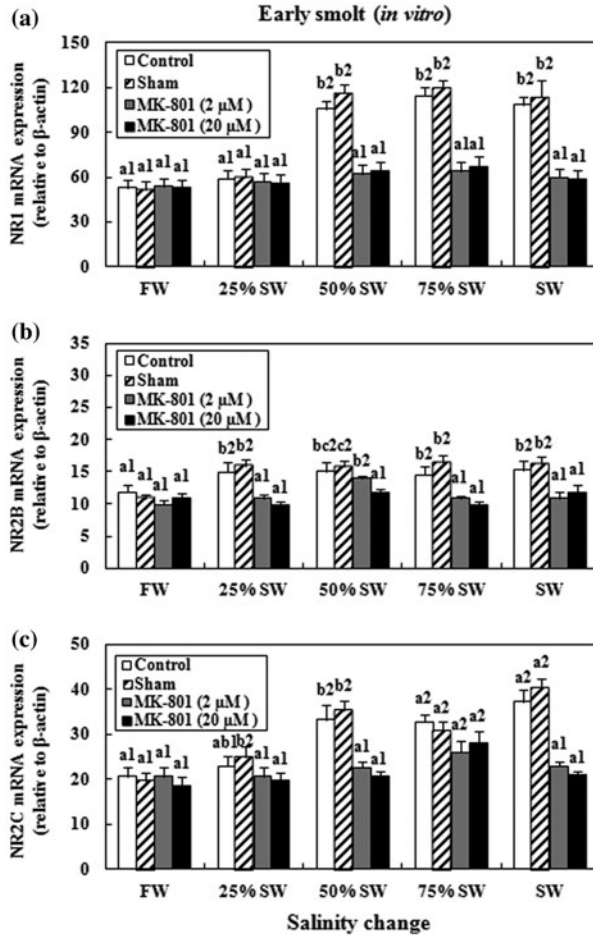
Figure 5.   mRNA expression levels of NRs in cultured brain cells of early smolt chum salmon (4 months after hatching) transferred from freshwater (FW, 0 psu) to seawater (SW, 35 psu) and after MK-801 treatment using quantitative real-time PCR.
Notes: Values are means ± SE (n = 15). Letters indicate significant differences between MK-801 treatment groups within the same salinity concentration. Numbers indicate a significant difference between salinity concentrations within the MK-801 treatment group ($p < 0.05$).

Bottai et al. (1997) and Harvey-Girard et al. (2007) also observed a high expression of NR1 and NR2 in the telencephalon of the knifefish *Apteronotus leptorhynchus*.

The mRNA expression of NRs increased with salinity, showing that salmon fry adapt to salinity conditions during PST. Yu et al. (2014) reported that an increase in NRs gene expression affected the memory capacities of chum salmon to move downstream. This suggests that NRs may play an important role on the migration between the sea and the natal stream.

To understand the role of MK-801, an antagonist of NRs, on growth of chum salmon fry, MK-801 (2 and 20 μM) was added to the different treatments. The mRNA expression of NRs was significantly reduced in all growth stages (early parr, parr, and early smolt stages), and this decrease was higher in older stages. These results corroborated the findings of Langton et al. (2007), who reported that MK-801 caused a faster
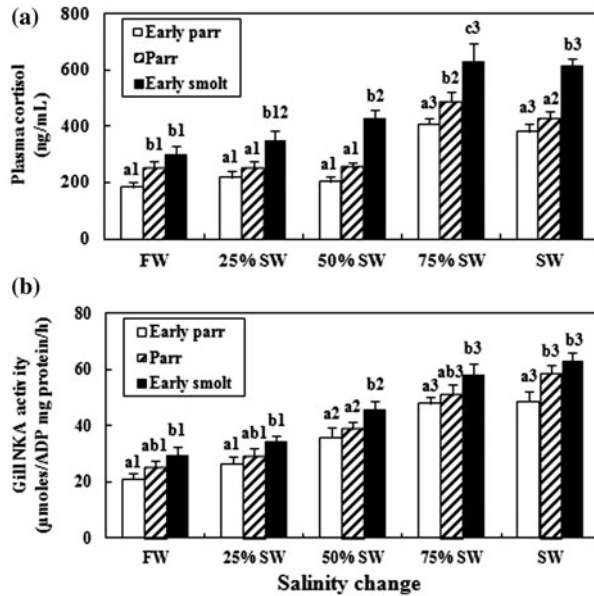
Figure 6. Levels in the plasma cortisol (a) and gill NKA activity (b) of early parr, parr, and early smolt stages of chum salmon (2, 3, and 4 months after hatching) transferred from freshwater (FW, 0 psu) to seawater (SW, 35 psu).
Notes: Letters indicate significant differences between PST period groups within the same salinity concentration. Numbers indicate a significant difference between salinity concentrations within the same PST period group ($p < 0.05$).

decrease in NRs on 24 days-old rats than on 17 days-old ones, and that NRs had a greater influence on late growth stages than on the initial ones. Results of the present study confirm that MK-801 is an antagonist of the NR in chum salmon, directly reducing the expression of NR genes. This means that the effect of MK-801 on the growth of juvenile larger received MK-801 treatment at progresses. In this experimental result, it was meant to undergo much more effect of NR (from parr stage to smolt stage) during growth in the salmon fry. Furthermore, the expression of NRs increased only during intermediate salinity levels, and this increase was only significant in parr and early smolt stages, suggesting that olfactory memory and imprint are more important in these stages.

The levels of plasma cortisol, T_4, T_3 and gills NKA activity increased gradually in all stages of salmon fry from FW to SW and were significantly higher at the early smolt stage. Cortisol is a corticosteroid hormone affecting the absorption of ions and water from the gills, and controlling the increase in body, glucose concentration and osmotic regulation (McCormick 2001; Martinez et al. 2005). Choi et al. (2013) have reported that plasma osmolality levels in the sockeye salmon *Oncorhynchus nerka* fry significantly increased during exposure to salinity changes (from FW to SW) and gained the ability to adjust osmolality to saline environments during smoltification. According to Dickhoff (1989), cortisol may act on olfactory receptors present in the brain and is directly involved on the olfactory imprinting and the long-term memory storage. Hasler and Scholz (1983) and Dickhoff et al. (1990) also reported that high levels of cortisol and thyroid hormones clearly influence the olfactory imprinting of the natal stream
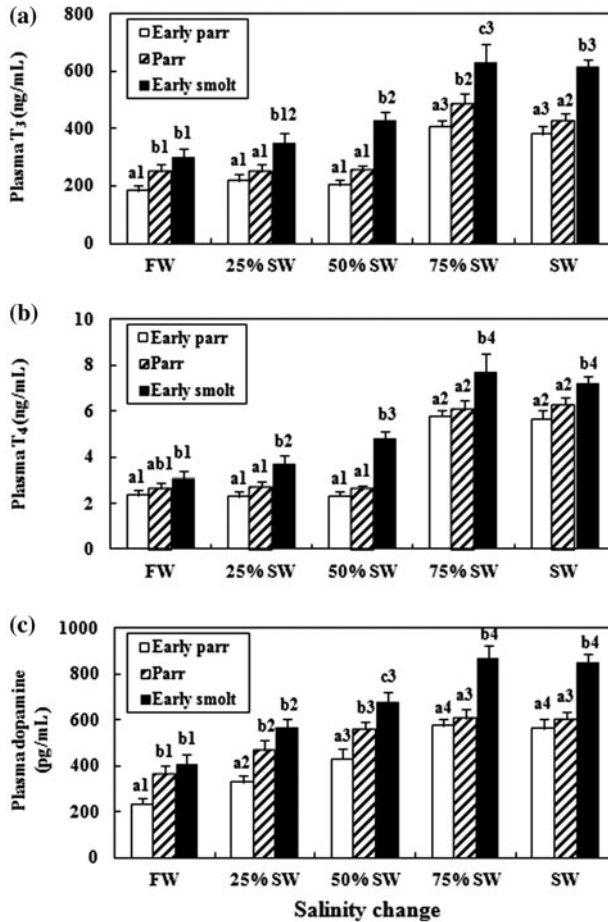
Figure 7. Plasma T_3 (a), T_4 (b), and dopamine (c) levels in early parr, parr, and early smolt stages of chum salmon (2, 3, and 4 months after hatching) transferred from freshwater (FW, 0 psu) to seawater (SW, 35 psu).
Notes: Letters indicate significant differences between PST period groups within the same salinity concentration. Numbers indicate a significant difference between salinity concentrations within the same PST period group ($p < 0.05$).

during PST. In this study, plasma cortisol and thyroid hormone levels increased during salinity changes, affecting osmoregulation and the olfactory memory, and were significantly higher at the early smolt stage. We suggest that olfactory memory promotes the adaptive capacity of chum salmon during PST smoltification, depending on the growth stage.

While a number of ion transporters are involved in ion extrusion in SW, NKA is essential for driving this process (McCormick 2001). Mancera and McCormick (2000) and Singer et al. (2003) have reported an increase in NKA activity when mummichog *Fundulus heteroclitus* and Atlantic salmon, *S. salar*, were transferred from FW to SW. This result indicates that fish maintain osmolality, and hence homeostasis, by excreting ions in the gill. In addition, NKA consists of α/β protein complex and the ion pump in chloride cells (Hootman & Philpott 1979); consequently, NKA mRNA and protein

activity increase to maintain homeostasis in response to salinity changes (McCormick 1995; Uchida et al. 1996). We suggest that gills NKA activity increases in order to maintain homeostasis during SW acclimation.

The levels of dopamine in the plasma increased during adaptation to SW. In particular, plasma dopamine levels were significantly higher in the early smolt stage of salmon fry. Dopamine is a neurotransmitter involved, in the mammalian brain, in the learning ability of olfactory and long-term storage memories (Hsia et al. 1999; Pignatelli et al. 2005). Knapp et al. (1990) reported that the presence of L-glutamate increased dopamine levels. 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 eel. In the present study, dopamine also increased with salinity and with fry growth from early parr to early smolt. The increase in dopamine and NR expression levels (especially in the early smolt stage) affected the imprinted olfactory memory and improved the long-term storage capability of the salmon fry brain.

We, therefore, confirmed that memory-related genes and hormones increased during growth, from parr to early smolt stage. In addition, we have confirmed that the expression of NRs affects the olfactory memory and learning, as observed during the MK-801 treatment. Therefore, this study provides not only important information on the mechanisms associated with NR expression and the formation of olfactory memories in salmon, but may also help to determine the best discharge time for salmon (between parr and early smolt), thereby improving the rate of return to natal streams.

Disclosure statement

Funding

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