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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,