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REVIEW PAPER

Molecular biological research on olfactory chemoreception in fishes

H. Hino*, N. G. Miles*, H. Bandoh*† and H. Ueda*†‡

**Laboratory of Aquatic Bioresources and Ecosystem, Field Science Center for Northern Biosphere, Hokkaido University, Sapporo, Hokkaido 060-0809, Japan and †Division of Biosphere Science, Graduate School of Environmental Science, Hokkaido University, Sapporo, Hokkaido 060-0809, Japan*

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This review describes recent molecular biological research on olfactory chemoreception in fishes. The recent rapid development of molecular biological techniques has provided new valuable information on the main and vomeronasal olfactory receptor (OR) genes, the axonal projection from ciliated, microvillous and crypt-olfactory receptor cells to the olfactory bulb, properties of odorant substances and olfactory imprinting and homing in salmon. Many important questions, however, remain unanswered on functional differences among OR genes, on ligand binding to each OR and on the molecular biological mechanisms underlying olfactory imprinting and homing in salmon. Olfactory chemoreception is believed to be the oldest sensory cue for both animal survival and adaptation to various different environments. Further intensive molecular biological research on olfactory memory formation and remembrance should be carried out to clarify the fundamental process of olfactory chemoreception in fishes.

Key words: homing; imprinting; olfactory chemoreception; olfactory receptor; salmon.

INTRODUCTION

The olfactory system of fishes is capable of recognizing and discriminating a large number of odours in the surrounding waters. Although fish inhabit an aquatic environment, their olfactory systems share many common characteristics with those of terrestrial vertebrates. In fishes, the discrimination of water-soluble odours is very important for several life functions such as feeding, reproduction, kin recognition, escape from danger and migration. To accomplish these functions, the olfactory system of fish detects many odorant substances including amino acids, nucleotides, steroids, prostaglandin and bile acids. This olfactory chemoreception is accomplished through binding of the odorant substance to an olfactory receptor (OR) in the olfactory epithelium with subsequent propagation of the information to the central nervous system.

‡Author to whom correspondence should be addressed. Tel–fax: +81 11 706 2598; email: hueda@fsc.hokudai.ac.jp

The OR genes that are expressed in both olfactory and vomeronasal epithelia belong to the vast enormous superfamily of G-protein-coupled receptors (GPCR) in vertebrates (Lancet & Pace, 1987; Reed, 1990; Buck & Axel, 1991). Volatile odours and odorant substances dissolved in water are detected by two types of olfactory receptor in the olfactory epithelium of vertebrates: namely, main olfactory receptors (MOR), which are expressed in ciliated olfactory receptor cells (cORC); and vomeronasal olfactory receptors (VOR), which are expressed in microvillous olfactory receptor cells (mORC). In mammals, it is believed that pheromones are received mostly by mORCs in the vomeronasal organ. Fish, however, lack the vomeronasal organ, so mORCs are distributed in the olfactory epithelium together with cORCs. In fish, the olfactory epithelium is composed of cORCs, mORCs, crypt ORCs, ciliated non-sensory cells, basal cells, supporting cells and goblet cells.

In general, vertebrate olfactory transduction initiates ligand binding to ORs, which then activates a heterotrimeric G-protein containing the olfactory-specific α-subunit, which in turn, activates type III adenylate cyclase to produce cAMP from ATP (Restrepo *et al*., 1996). The increase in intercellular levels of cAMP activates an olfactory-specific cyclic nucleotide-gated channel (CNG channel), which is a non-selective cation channel. The influx of sodium and calcium through the activated channel depolarizes ORCs. In addition, the calcium ion influx through the channel activates calcium-activated chloride channels, leading to chloride efflux and further depolarization. In goldfish *Carassius auratus* (L.), cORCs have been shown to express CNG channels (Hansen *et al*., 2004). Although the subsequent transduction cascade that is activated in mORCs is unclear, a phospholipase C (PLC)-mediated pathway has been suggested in zebrafish *Danio rerio* (Hamilton) (Ma & Michel, 1998). The PLC-mediated pathway includes amino acid odorant-stimulated release of IP3 and diacylglycerol (DAC) in channel catfish *Ictalurus punctatus* (Rafinesque) (Restrepo *et al*., 1993). Although the functional role of crypt ORCs remains unknown, they have both cilia and microvili in their crypt, and may express two different G-proteins (Hansen & Zielinski, 2005). Furthermore, the presence of a cAMP transduction pathway that might transduce odorants such as amino acids has been reported in crypt ORCs of Pacific jack mackerel *Trachurus symmetricus* (Ayres) (Vielma *et al*., 2008).

In mammals, the recognition of odours is important to escape from predators, to search for food, to avoid consumption of toxic products and to find breeding partners for reproduction. The memorization of such olfactory information is crucial for animal survival. Salmon are well known for their accurate homing migration, which is guided by odours imprinted during downstream migration from their natal stream to the open water. Early behavioural studies demonstrated that coho salmon *Oncorhynchus kisutch* (Walbaum) are unable to return to their natal stream if their olfactory sense is blocked during their homing migration, suggesting that olfaction is crucial for homing migration (Wisby & Hasler, 1954). Those studies led to the olfactory hypothesis that salmon memorize the odours of natal stream water during their juvenile river life (olfactory imprinting) and the adults return to the same natal stream by recalling memories of their natal stream odours (olfactory homing) (Hasler & Scholz, 1983).

With the recent rapid development of molecular biological technology, many studies have examined olfactory chemoreception in vertebrates from several aspects, including genomic organization, regulation of expression and receptor function.

Many of these studies have provided crucial information for understanding how the olfactory system recognizes and distinguishes thousands of odours. In this review, the authors discuss the molecular structure of putative MOR and VOR genes in fish, properties of odorant substances in fish, the axonal projection from ORCs to the olfactory bulb and investigations of the olfactory memory in fish, with particular emphasis on the olfactory imprinting and homing of salmon, as determined using molecular biological techniques.

MAIN OLFACTORY RECEPTOR AND VOMERONASAL OLFACTORY RECEPTOR GENES

The first MORs were identified in the rat *Rattus novegicus*, and are now referred to as the OR superfamily (Buck & Axel, 1991; Mombaerts, 2004). The predicted structure of these receptors exhibits a seven transmembrane domain topology characteristic of GPCRs. The OR gene superfamily is the largest multigene superfamily so far described in mammalian genomes. For example, analysis of whole-genome sequences has shown that there are 800 human MOR genes, of which *c*. 50% are pseudogenes (Glusman *et al*., 2001; Niimura & Nei, 2003). Furthermore, completion of the mouse *Mus musculus* genome confirmed the existence of *c*. 1400 potential MOR genes of which *c*. 25% are pseudogenes (Young *et al*., 2002; Zhang & Firestein, 2002; Zhang *et al*., 2004*a*; Niimura & Nei, 2005). MOR genes have also been identified in several fish species, including *I. punctatus* (Ngai *et al*., 1993*b*), *D. rerio* (Weth *et al*., 1996; Barth *et al*., 1997; Dugas & Ngai, 2001), *C. auratus* (Cao *et al*., 1998), lamprey *Lampetra fluviatilis* (L.) (Freitag *et al*., 1999), medaka *Oryzias latipes* (Temminck & Schlegel) (Sun *et al*., 1999; Kondo *et al*., 2002), Japanese loach *Misgurnus anguillicaudatus* (Cantor) (Irie-Kushiyama *et al*., 2004) and a number of salmonids (Wickens *et al*., 2001; Dukes *et al*., 2004, 2006; Morinishi *et al*., 2007). The sequence phylogeny of fish MORs is shown in Fig. 1. Although the exact numbers of MOR genes are unknown, molecular cloning and genomic DNA blot hybridizations in fish species suggest an MOR repertoire size that is approximately five-fold to ten-fold smaller than that of mammalian species (Alioto & Ngai, 2005; Niimura & Nei, 2005).

Almost all vertebrate MOR genes have a single intronless coding exon of *c*. 1 kb (Mombaerts, 1999), and have seven transmembrane domains (TMD 1–7) and a unique amino acid sequence (Fig. 2). The most conserved regions of MORs across several species occur in the second intracellular and extracellular loops as well as within TMD 2, TMD 6 and TMD 7. On the other hand, TMD 3, TMD 4 and TMD 5 exhibit striking divergence (Zhao & Firestein, 1999). A wide variety of odorant molecules probably bind to sites in TMD 3, TMD 4 and TMD 6 (Ngai *et al*., 1993*a*). Most MOR genes have a few consensus landmark motifs. These consensus motifs have been identified as unique to MORs in mammals (Zhao & Firestein, 1999), and include a mayDRyvAiCxPLxY motif (capital letters indicate highly conserved amino acid residues) at the C-terminal of TMD 3 and the second intracellular loop; and a KaFsTCxsh motif at the N-terminal of TMD 6. The cysteine motifs found in all GPCRs are conserved at the first and second extracellular loops and at the beginning of TMD 7 (Gat *et al*., 1994). Four conserved cysteines lying within the second extracellular loop and between the second and third intracellular loops are

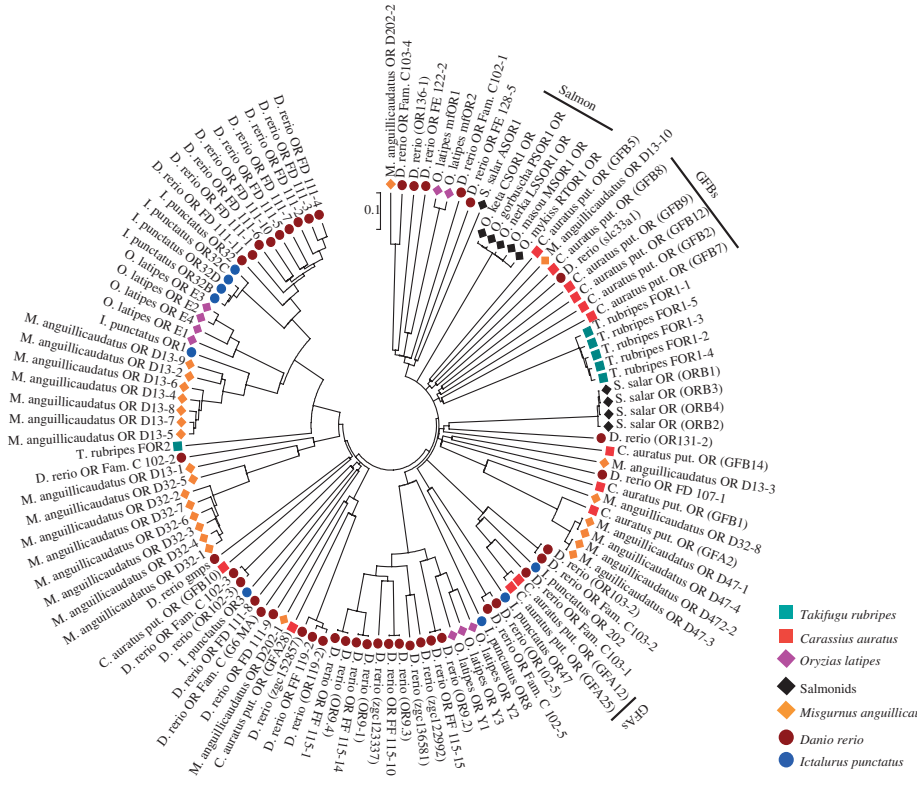

FIG. 1. Phylogenetic tree of putative fish olfactory receptor (OR) genes. The phylogenetic tree is based on the sequence of six *Takifugu rubripes*, 13 *Carassius auratus*, nine *Oryzias latipes*, 10 salmonids, 24 *Misgurnus anguillicaudatus*, 42 *Danio rerio*, and nine *Ictalurus punctatus* OR genes, and was constructed by MEGA4 (http://evolgen.biol.metro-u.ac.jp/MEGA/).

believed to form two disulphide bridges that are unique to MORs, suggesting that these disulphide bridges could be important for enhancing structural stability of MOR proteins (Sharon *et al*., 1998).

Vertebrate MOR genes can be classified into two different groups, termed class I and class II. Fish MOR genes are exclusively class I genes, except for a few genes in the coelacanth *Latimeria chalumnae* Smith (Freitag *et al*., 1998). Tetrapods have both classes of genes (Niimura & Nei, 2005), and a whole field of study has arisen to look at the different expression patterns and targeting instructions of mammalian class I and II genes (Bozza *et al*., 2009). Interestingly, the African clawed frog *Xenopus laevis* has both types of gene: class I genes are exclusively expressed in the lateral diverticulum of the nose, which detects water-soluble odours, whereas class II genes are expressed in sensory neurons of the diverticulum that detects volatile odours (Freitag *et al*., 1995). Moreover, Zhang *et al*. (2004*b*) indicated that the olfactory epithelium of mouse has different zones expressing class I and class II genes, suggesting a functional difference of each zone. Although class I MORs might recognize different types of ligand from those of class II MORs (Zhang &

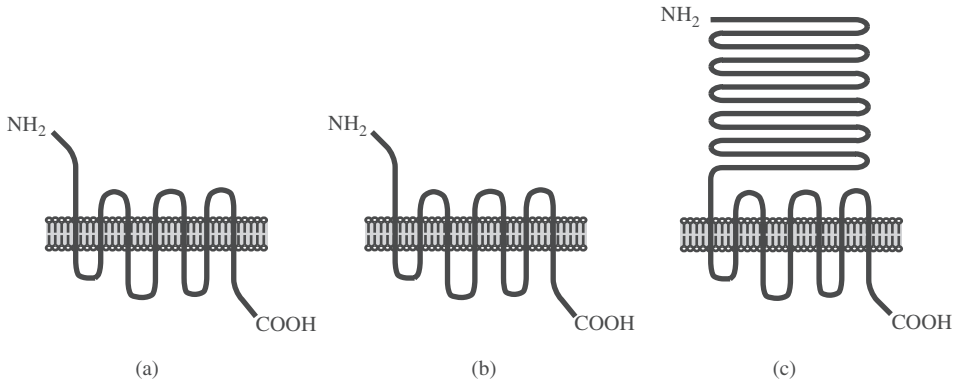

FIG. 2. Schematic illustration of main olfactory receptors (MOR) and vomeronasal olfactory receptors (VOR). (a) MORs, (b) V1Rs, (c) V2Rs.

Firestein, 2002), the functional difference between class I and II genes is currently unclear.

Mammalian VORs belong to one of the two families of receptor genes, known as the V1R (Dulac & Axel, 1995) and V2R (Herrada & Dulac, 1997; Matsunami & Buck, 1997; Ryba & Tirindelli, 1997) gene families. To date, *c*. 100 V1R genes have been identified in mammalian species (Rodriguez *et al*., 2002; Grus *et al*., 2005). It has recently been proposed that fish V1R-like genes should be called *ora* (olfactory receptors related to class A GPCRs) (Saraiva & Korsching, 2007) and V2R-like genes *OlfC* (olfactory receptors related to class C GPCRs) (Alioto & Ngai, 2006). Members of the V1R gene family have been found in a few fish species, including *D. rerio* (Pfister & Rodriguez, 2005), *O. latipes*, spotted green pufferfish *Tetraodon nigroviridis* (Marion de Procé) and Japanese pufferfish *Takifugu rubripes* (Temminck & Schlegel) (Saraiva & Korsching, 2007).

Approximately 60 V2R genes are present in the mouse and rat genomes (Yang *et al*., 2005). V2R genes also have been identified in non-mammalian vertebrates such as *T. rubripes* (Naito *et al*., 1998), *C. auratus* (Cao *et al*., 1998), *D. rerio* (Alioto & Ngai, 2006), Atlantic salmon *Salmo salar* L. (Dukes *et al*., 2006) and African clawed frogs (Hagino-Yamagishi *et al*., 2004). The percentage of pseudogenes among the V2R genes in fish ranges from 26 to 47% (Hashiguchi & Nishida, 2006). The sequence phylogeny of fish VORs is shown in Fig. 1. The V2R receptors belong to the C family of GPCRs, which includes metabotropic glutamate receptors (mGluR), extracellular calcium sensing receptors (CaSR) and γ-amino butyric acid $(GABA)_B$ receptors (Pin *et al*., 2003).

VORs encode seven transmembrane domain protein receptors, which are likely to be coupled to G-proteins. Like MOR genes, V1R genes contain ligand-binding pockets in the transmembrane domain. In contrast, V2R genes differ from V1R and MOR genes by having a long N-terminal extracellular domain that contains the primary determinants of ligand binding (Okamoto *et al*., 1998; Han & Hampson, 1999) (Fig. 2). V1Rs and class II ORs have been suggested to bind to small airborne chemicals, whereas V2Rs and class I ORs recognize water-soluble molecules;

in support of this hypothesis, Shi & Zhang (2007) have demonstrated an evolutionary shift of VR gene repertoires in the vertebrate transition from water to land.

THE AXONAL PROJECTION FROM OLFACTORY RECEPTOR CELLS TO THE OLFACTRORY BULB

In teleosts, the axonal projection from cORCs and mORCs to the olfactory bulb has been studied by retrograde tracing experiments in which DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate) is injected into various regions of the olfactory bulb to trace back to the originating cell bodies of ORCs in the olfactory epithelium (Morita & Finger, 1998; Hansen *et al*., 2003). In *I. punctatus*, cORCs project into the medial and ventral regions of the olfactory bulb, whereas the dorsal region of the olfactory bulb tends to be innervated by mORCs (Morita & Finger, 1998; Hansen *et al*., 2003). In the crucian carp *Carassius carassius* (L.), the axonal projections of cORCs and mORCs are observed in the medial and lateral part of the olfactory bulb, respectively (Hamdani & Døving, 2002). The axonal projections of cORCs and mORCs to the olfactory bulb, however, have also been investigated in transgenic *D. rerio* by using cell type-specific promoter elements (Sato *et al*., 2005). In that study, the expression patterns of receptors and ion channels such as MOR, V2R, transient receptor potential channel C2 and olfactory marker protein (OMP) were examined in transgenic *D. rerio*, showing that the axons of cORCs project to the dorsal and medial part in the olfactory bulb, whereas those of mORCs project to the lateral part. Moreover, the same group has proposed a model of the hierarchical regulation of OR gene choice and subsequent axonal projection in the *D. rerio* olfactory system (Sato *et al*., 2007).

PROPERITES OF ODORANT SUBSTANCES

In teleosts, olfactory responses to odorant substances dissolved in water such as amino acid and pheromone-like substances have been recorded by using electrophysiological techniques, as well as calcium imaging techniques (Friedrich & Korsching, 1998). Erickson & Caprio (1984) reported that both the cORCs and mORCs of *I. punctatus* respond well to amino acids and bile acids. Moreover, Zippel *et al*. (1997) reported that mORCs of *C. auratus* preferentially detect bile acids, steroids and prostaglandins. Sato & Suzuki (2001) measured the whole-cell response of cORCs and mORCs in rainbow trout *Oncorhynchus mykiss* (Walbaum) to amino acid and pheromone candidate substances, such as 17α,20β-dihydroxy-4-pregnen-3-one, etiocholan-3-ol-17-one glucuronide, prostaglandins and urine, by whole-cell voltage-clamp techniques, suggesting that cORCs respond to a wide variety of odorants, including pheromones, whereas mORCs respond only to amino acids. Using an expression cloning technique in *Xenopus* oocytes, Speca *et al*. (1999) isolated a cDNA encoding a *C. auratus* OR, GFA 5.24, that is activated by amino acids. GFA 5.24 is activated by arginine and lysine and interacts with these compounds with high affinity, sharing sequence similarities with CaSRs, mGluRs and the V2R class

of VORs. Moreover, Lipschitz & Michel (2002) observed the distribution of ORCs stimulated by amino acids by using a cation channel-permeant probe, agmatine, and showed that amino acids stimulate at least mORCs in *D. rerio*. Collectively, these reports suggest that V2Rs expressed in mORCs might detect amino acids but not pheromone-like substances. There is, however, one study reporting that amino acids are detected not only by mORCs but also by cORCs (Hansen *et al*., 2003).

Although many MORs and VORs have been identified from several vertebrates owing to the progress of whole-genome analysis, many ligands remain uncharacterized. Moreover, new candidates for pheromone-like substances have been isolated by Yambe *et al*. (2006) who, using electrophysiological and behavioural analysis, showed that the sex pheromone L-kynurenine in the urine of ovulating female masu salmon *Oncorhynchus masou* (Brevoort) could be detected only by precocious spermiating males. Using electrophysiological analysis, Shoji *et al*. (2000) showed that the olfactory organ of *O. masou* can distinguish variations in the composition of amino acids in different river waters. In addition, Liberles & Buck (2006) have reported that the trace amine-associated receptor (TAAR) family expressed in mouse olfactory neurons recognizes volatile amines, suggesting that a function of TAAR is associated with the detection of social cues. Interestingly, TAAR gene sequences are also present in *D. rerio*, according to the analysis of genome sequence data (Gloriam *et al*., 2005), but it is unclear which substances are recognized by fish TAARs.

OLFACTORY IMPRINTING AND HOMING IN SALMON

Pacific salmon show differences in the timing of downstream and upstream migration (Fig. 3). Chum salmon *Oncorhynchus keta* (Walbaum) and pink salmon *Oncorhynchus gorbuscha* (Walbaum) have juveniles that start downstream migration for the ocean immediately after emergence. These fish grow to maturity in the North Pacific Ocean, after which the adult salmon start their homing migration. They migrate upstream from the North Pacific Ocean to their natal river for spawning several weeks before gonadal maturation. Sockeye salmon *Oncorhynchus nerka* (Walbaum) *O. masou* and *O. kisutch* have juveniles that spend *c*. 16–18 months in the natal river, during which individuals obtain seawater tolerance by means of the smoltification or parr–smolt transformation (PST); they then start downstream migration towards the ocean or lake. Adult salmon migrate upstream to their natal river several months before gonadal maturation.

Two different olfactory hypotheses have been proposed for salmon imprinting and homing: one is the imprinting hypothesis developed by Wisby & Hasler (1954) using *O. kisutch*; the other is the pheromone hypothesis developed by Nordeng (1971, 1977) using Arctic char *Salvelinus alpinus* (L.) and *S. salar*. The pheromone hypothesis assumes that juvenile salmon in a stream release population-specific odours that guide homing adults. There are, however, no juveniles of *O. keta* or *O. gorbuscha* present at the time that the adults home to their natural stream. It is now widely accepted that some specific odorant factors in the natal stream are imprinted on the olfactory system of juvenile salmon during downstream migration, and that adult salmon evoke these factors to recognize their natal stream during homing migration (Dittman & Quinn, 1996; Quinn, 2005; Ueda *et al*., 2007).

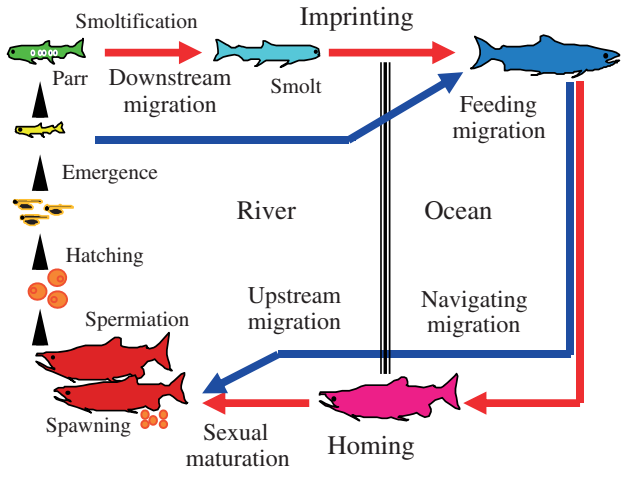

FIG. 3. Schematic illustration of the life history of Pacific salmon. Blue lines indicate *O. keta* and *O. gorbuscha*. Red lines indicate *O. nerka*, *O. masou* and *O. kisutch*.

Harden Jones (1968) and Brannon (1982) proposed that juvenile Pacific salmon learn a series of olfactory waypoints during their migration through fresh water, and subsequently adult salmon retrace this odour sequence during homing migration. There are, however, few detailed reports on the timing of the olfactory imprinting of Pacific salmon. By using artificial odorants, Hasler & Scholz (1983) suggested that juvenile *O. kisutch* learn the odours of their home stream during the PST. In addition, Nevitt *et al*. (1994) demonstrated in *O. kisutch* that olfactory receptor cells isolated from 6 to 9 month-old fish after β-phenylethyl alcohol (PEA) imprinting during the PST retained high sensitivity to the imprinted PEA odour by using the whole-cell patch-clamp technique. Dittman *et al*. (1996) also confirmed the importance of the PST as a sensitive period for olfactory imprinting in this species.

The formation of memory has been intensively studied in mammals by both molecular biological and electrophysiological techniques. Recently, many of the relevant experimental studies have concentrated on the possible role of long-term potentiation (LTP) in learning and memory, with a focus on the *N*-methyl-D-aspartate (NMDA) receptor that induces LTP. LTP is known to occur in the brain of lacustrine (landlocked) *O. nerka* (Satou *et al*., 1996), *D. rerio* (Nam *et al*., 2004), *O. mykiss* (Kinoshita *et al*., 2004) and common carp *Cyprinus carpio* L. (Satou *et al*., 2006). In addition, NMDA has also been reported to be expressed in the brain of lacustrine *O. nerka* (Fukaya, 1999) and *O. mykiss* (Matsuoka *et al*., 1998; Kinoshita *et al*., 2005). These findings support the possibility that olfactory imprinting and homing in salmon is controlled at the gene level, and that the genes related to both processes are expressed in the olfactory system of salmon.

SALMON OLFACTORY SYSTEM-SPECIFIC PROTEIN

There have been several attempts to investigate olfactory imprinting and homing mechanisms in salmon by biochemical and molecular biological techniques. The salmon olfactory system-specific protein N24 was identified in lacustrine *O. nerka* by

an electrophoretic comparison, using sodium dodecyl sulphate–polyacrylamide gel electrophoresis, between proteins restricted to the olfactory system and those found in other parts of the brain (Shimizu *et al*., 1993). A polyclonal antiserum specific for N24 recognized only a 24 kDa protein in the olfactory system, as determined by western blot analysis. Furthermore, N24 immunoreactivity was found in the olfactory system of teleost species that migrate between sea and river, such as Japanese eel *Anguilla japonica*, Temminck & Schlegel, but not in non-migratory species, such as *C. carpio* (Ueda *et al*., 1994). Interestingly, N24 immunoreactivity has also been observed in the testicular germ cells, spermatids and spermatozoa, suggesting its involvement in sperm chemotaxis (Ueda *et al*., 1993). Immunocytochemical and immunoelectron microscopic observations showed that N24 positive immunoreactivity was present in cORCs and mORCs, and in the glomerular layer near the mitral cells in the olfactory bulb (Kudo *et al*., 1996*b*). A cDNA encoding N24 has been isolated and sequenced, and this cDNA contains a coding region corresponding to 216 amino acid residues. Protein and nucleotide sequencing demonstrated the existence of homology between N24 and glutathione *S*-transferase (GST; EC 2.5.1.18) class pi enzymes (Kudo *et al*., 1999). Northern blot analysis showed that N24 mRNA of 950 base pairs (bp) is expressed in lacustrine *O. nerka* olfactory epithelium. The functional roles of N24 during salmon homing migration are still unclear, but N24 is thought to be involved both in olfactory termination by xenobiotic function in the olfactory receptor cells and in neuromodulation in the glomeruli of salmon (Kudo *et al*., 1996*a*, 1999).

SALMON OLFACTORY IMPRINTING-RELATED GENE

In a study to identify olfactory imprinting-related genes, Hino *et al*. (2007) recently isolated SOIG [(*O. nerka*) salmon olfactory imprinting-related gene] from the olfactory system of lacustrine *O. nerka* by subtractive hybridization technique of cDNA-representational difference analysis (cDNA-RDA) using fish at the PST as a tester and fish at the feeding migration term as a driver. By northern blot analysis and *in situ* hybridization using several tissues of lacustrine *O. nerka* at the PST, SOIG mRNA was shown to be expressed in olfactory receptor cells and basal cells of the olfactory epithelium. The open reading frame of SOIG cDNA contains two Ly-6 superfamily domains. Although the function of most of the Ly-6 superfamily remains unclear, that of some genes is known. Of particular interest, the Ly-6-related protein (*odr-2*), which contains two Ly-6 superfamily domains, isolated from the nematode *Caenorhabditis elegans* has been suggested to regulate olfactory neuron signalling within the neuronal network required for chemotaxis (Chou *et al*., 2001). The expression levels of SOIG mRNA in the olfactory epithelium have been analysed during several life-cycle stages of lacustrine *O. nerka* and *O. keta*, such as ontogeny, PST and homing (Hino, 2007). During ontogeny, the expression levels of SOIG mRNA are significantly higher in alevin (juvenile fry) than in embryos at 43 and 60 days after fertilization, and then they surge at the PST in lacustrine *O. nerka*. On the other hand, SOIG mRNA levels in the olfactory epithelium of *O. keta* during homing migration are elevated at the estuary and prespawning ground. The function of SOIG during the PST and homing migration of salmonids is still unknown, but it is thought that it might be related to olfaction or cell proliferation during both the PST and the final stage of homing.

OLFACTORY IMPRINTING IN OTHER ANIMALS

Olfactory imprinting phenomena are also found in other animals. For example, Harden *et al*. (2006) demonstrated that *D. rerio* can be imprinted with PEA. A gene, *otx2*, upregulated in the olfactory epithelium of PEA-imprinted fish was then isolated, and its expression was compared with that in non-PEA-imprinted fish using *D. rerio* oligo expression array chips. *Otx2* was shown by *in situ* hybridization to encode a transcription factor that is upregulated in olfactory sensory epithelia in response to PEA, and an increase in the expression of *otx2* was found at 2–3 days post-fertilization and maintained in the adult animals. Interestingly, the expression of *otx2* in response to other odorants such as L-cysteine and vanilla did not increase, suggesting that the increase in *otx2* expression was not a generalized response to chemicals added to the water. By double *in situ* hybridization using several neuron makers, *otx2* was found to colocalize with *notch1a*, which encodes a transcription factor expressed in a neuronal precursor in the developing olfactory epithelium, and *HuC*, encoding a marker for differentiating neurons; these findings suggest that *otx2* plays a role in the specification of a pool of olfactory sensory neurons in the developing *D. rerio* olfactory epithelium. The detailed function of *otx2* in relation to olfactory imprinting in *D. rerio* is, however, not yet understood because the *otx2* gene encodes an essential transcription factor that is widely expressed in the developing embryo from epiboly, and loss of this gene function is lethal.

In order to elucidate the function of unidentified genes, many experiments based on RNA interference and deletion methods have been performed. For example, Remy & Hobert (2005) examined the effects of downregulation of the G-protein-coupled chemoreceptor family member gene, *sra-11*, which is expressed in the interneuron of odour-imprinted *C. elegans*, and showed that the function of this chemoreceptor family member gene is related to the cellular and molecular basis of olfactory imprinting. Moreover, it has been reported that OMP, which is a marker for mature olfactory sensory neurons, contributes to olfactory sensitivity in studies based on an electrophysiological technique using OMP-knockout mice (Buiakova *et al*., 1996; Ivic *et al*., 2000; Youngentob *et al*., 2001). These techniques would be particularly beneficial for future research into elucidation of the function of SOIG and N24 which may play important roles in the life cycles of fishes.

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