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Abstracts of the 32nd Annual Meeting and the 32nd Symposium on
"Progress in Comparative Endocrinology for 10 Years
and Perspectives in Future",
and
The 8th Joint Symposium of Three Japanese Societies of Comparative
Biology on "Comparative Biology of Regeneration"

Nikko
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Edited by

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S-1

HORMONAL REGULATION OF OOCYTE MATURATION IN TELEOSTS AND STARFISH - COMPARATIVE TO GENERAL -

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Oocyte maturation is the process by which the oocyte completes the first meiotic division and undergoes several maturational changes, and progresses to metaphase II. Studies from our laboratory using teleosts and starfish have indicated that oocyte maturation is regulated by three major mediators, namely gonadotropin (LH in teleosts and gonad-stimulating substance, GSS in starfish), maturation-inducing hormone (MIH), and maturation-promoting factor (MPF).

In fish, LH acts on ovarian follicle cells to produce fish MIH (17α, 20β-dihydroxy-4-pregnen-3-one, 17α, 20β-DP). 17α, 20β-DP is synthesized by a two-step process involving the thecal and granulosa cell layers. A novel type of P450c17 lacking the lyase activity is responsible for the shift from estradiol-17β to 17α, 20β-DP in ovarian follicles occurring immediately prior to oocyte maturation. We recently purified GSS from the radial nerves of starfish (*Asterina pectinifera*). The purified GSS is a heterodimeric peptide composed of A- and B-chains, with disulfide cross-linkages, and was named “genitalin” as a novel insulin-like neuropeptide hormone (Mita *et al*., unpublished). Genitalin acts on ovarian follicle cells to produce starfish MIH (1-methyladenine, 1-MeAde).

The site of 17α, 20β-DP and 1-MeAde is the surface of the oocytes. A distinct family of membrane-bound progestin receptors having characteristics of G-protein-coupled receptors seems to mediate non-genomic actions of 17α, 20β-DP. The MIH signal received on the oocyte surface is transduced into the oocyte cytoplasm for the formation and activation of MPF, the final mediator of oocyte maturation. Although MPF exhibits a universal molecular structure as a complex of cdc2 and cyclin B in any species, the mechanisms of MPF formation and activation have been modified to various extents from species to species. In fish, the 17α, 20β-DP signal leads to the *de novo* synthesis of cyclin B, the regulatory component of MPF, which activates a preexisting 35-kDa cdc2 kinase via phosphorylation of its threonine 161 by a threonine kinase, thus producing the 34 kDa active cdc2.

In summary, our findings appear to show that the basic mechanisms involved in oocyte maturation are the same in vertebrates (teleosts) and invertebrates (starfish), despite the differing chemical nature of the hormonal agents involved.

S-2

NEUROENDOCRINOLOGY OF INSTINCTIVE BEHAVIOR TOLD BY ANURAN AMPHIBIANS AND SALMONIDS

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Instinctive behavior is defined as that for survival of individuals and maintenance of species, and is considered to be genetically programmed in the genome. In vertebrates, the neuroendocrine systems located in the hypothalamus are involved in the controls of almost all types of instinctive behaviors. Then, how do hypothalamic neurons, in particular neurosecretory neurons (NSCs) which regulate endocrine functions, control instinctive behavior? Because of complex interactions between the higher brain center and the hypothalamus in higher vertebrates, a use of lower vertebrates, such as amphibians and teleosts, would be better to clarify general principles of neuroendocrine control mechanisms of instinctive behavior. Another advantage for the use of amphibian and teleost brains is availability of *in vitro* imaging analysis of a hemisected brain preparation.

The studies of calling behaviors in leopard frog (*Rana pipiens)* and Japanese toad (*Bufo japonicus*) indicated an interaction of NSCs with the activating reticular systems. When a leopard frog was acoustically stimulated with conspecific mating calls, neurons in the anterior preoptic nucleus (APON), which is the triggering center of male mating behavior, were excited under the influence of injection of pituitary homogenate. Golgi-electron microscopic and retrograde HRP analyses, and immunocytochemistry showed that APON neurons receive various types of afferents including projections from neurons containing gonadotropin-releasing hormone (GnRH) and vasotocin. Iontophoretically applied GnRH and vasotocin immediately excited APON units, whereas intracerebroventricular (icv) GnRH enhanced amplitudes and frequencies of electroencephalogram almost 24 hrs after the treatment. Similar icv treatments with GnRH elevated the plasma testosterone levels also after considerable lag times. These results suggest dual controls by NSCs of the nervous system and the endocrine system to motivate instinctive behavior.

On the basis of abovementioned idea, we attempted to clarify neuroendocirne mechanisms of salmon spawning migration, or homing migration to the natal river. In the salmonid brain, GnRH and vasotocin neurons also send their immunoreactive fibers to various brain loci and the neurohypophysis, indicating that NSCs are involved in the dual control of nervous and endocrine systems in terms of motivation of instinctive behavior. Actually, application of GnRH analogue facilitated homing behavior, and enhanced expression of genes for gonadotropin subunits, in sockeye salmon. Thus, detailed quantitative analyses of molecular events in the neuroendocrine systems were carried out in aquacultured salmonids as model fishes and oceanic and homing chum salmon as wild fish. The molecular events seen in pre-spawning fishes, both model and wild, were similarly under the influence of the hypothalamus-pituitary-gonadal axis, however, the events in the high sea were additionally under the control of IGF-I, suggesting an importance of interaction between the neuroendocrine and the visceral systems.

S-3

IDENTIFICATION OF HORMONAL SIGNAL ESSENTIAL FOR MAMMALIAN HIBERNATION

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Mammals are characterized by maintaining a constant body temperature (Tb) near 37°C, by which high metabolic activities are kept independently of environmental changes. When Tb is decreased, principle organs, such as the central nervous system and hearts, dysfunction, resulting in death. However, hibernators have a unique ability to survive potentially lethal low Tb during the hibernation season. During hibernation, Tb is decreased below 10°C with a marked reduction of metabolic rate (about 100 times less than that in euthermic states), indicating that the ability to tolerate nearly arrested metabolic states is developed in association with hibernation. Furthermore, hibernation has been suggested to protect organisms from various lethal diseases and harmful factors. A factor that controls hibernation has long been explored, but not found. Here, a recent identification of hormonal factor essential for hibernation is summarized.

In chipmunks (*Tamias sibiricus*), a rodent hibernator, hearts normally function even at 5-6°C of body temperature only during the hibernation season. This was due to modulation of Ca^{2+} source for cardiac excitation-contraction coupling; Ca^{2+} source was changed from external Ca^{2+} influx to internal Ca^{2+} release of cytoplasmic store sites (1). This modulation was suggested to prevent lethal Ca^{2+} overload of myocardium induced at low Tb. This study led us to assume endogenous factors that modulate organisms for hibernation, then hibernation-specific protein (HP) complex was discovered in the blood (2). HP complex was produced in the liver and downregulated during the hibernation season by an endogenously generated circannual rhythm independently of Tb and environmental changes. In correlation with this downregulation, HP complex was transported into the brain through the blood-cerebrospinal fluid barrier and played an essential role in hibernation (3). Thus, HP complex carries an essential signal for hibernation from the liver to the brain under control of a circannual rhythm signaling.

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S

PROPOSING OF A NOVEL REGULATION SYSTEM OF THE ANTERIOR PITUITARY GLAND IN THE RAT

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It was demonstrated a few fluorescent LH-RH fibers in the pars tuberalis as well as on the primary plexus and in the ependymal layer. The distribution of the S-100 protein cell (folliculo-stellate cell) is very important to our understanding of the regulation of the anterior pituitary. The distribution of the cells observed over 50% of the total cell population in the so called “transitional zone”. They then decreased in number with distance from this region to mid-way towards the sagittal axis before increasing again in the tail of the gland. The population of cells also decreased with increasing distance from the "transitional zone" to the wing and with distance from the basal zone. Portal vessels entered the anterior lobe through the "transitional zone" as thick capillaries, ran through the basal surface of the anterior lobe. The distribution pattern of the folliculo-stellate cells and the capillaries showed good agreement and the spatial relationship between these two is detailed so as to better understand hypophyseal histophysiology.

Clusters of S-100 protein cells and numerous connexin 43 positive sites on S-100 protein cells were clear in the “transitional zone” as well as in the pars distalis. Random penetration of electrode showed that larger populations of cell (~80 %) had membrane potentials with - 55.6 ± 5.1 mV, and less than 20 % of cells had the resting membrane potential with - 36.0 ± 4.4 mV. There were two types of cell couplings; one major group for the recordings from cells with similar deep resting membrane potentials and the other for the recordings from cells with different resting membrane potentials. The former indicated that two cells were electrically coupled while the latter no electrical couples were observed. Carbenoxolone depolarized the membrane by 12.3 ± 5.5 mV and reduced the amplitude of electrotonic potentials, and the response recovered by removal of carbenoxolone by the superfusate.

The results clearly indicated that the folliculo-stellate cell system deeply participated in the regulation of the anterior pituitary parallel with the portal vessel system, which was the main regulatory system for pituitary hormone secretion.

P1

ANALYSIS OF THE RELATIONSHIP BETWEEN THE EXPRESSIONS OF AD4BP/SF-1 AND STEROIDOGENIC ENZYMES IN THE DEVELOPING GONADS OF A SQUAMATA SPECIES WITH TEMPERATURE-DEPENDENT SEX DETERMINATION

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Sex steroid hormones play an important role in sex determination and sex differentiation in squamate species with temperature-dependent sex determination, as well as in squamate species with genotypic sex determination. In the leopard gecko, *Eublepahris macularius*, it is known that estrogen treatment can make the sex of embryo female at any incubation temperature and we had reported female specific expression of estrogen synthetase, *P450arom* in the gonad of hatchlings. However, it remains unknown what mediates between the effect of incubation temperature and the sex steroid hormone signaling system in this species. *Ad4BP/SF-1* is one candidate for the mediator. This transcription factor is reported to regulate the expression of steroidogenic enzymes in the developing gonad of the mouse and chicken in sex specific manner. Thus, we tried to identify the cDNA of Ad4BP/SF-1 and conducted the expression analysis in the gonads of the leopard gecko embryos and hatchlings.

The length of the leopard gecko Ad4BP/SF-1 cDNA identified in this study was 1490 bp with open reading frame encoding a 468 amino acid protein. The deduced amino acid sequence showed moderate identity with that of other vertebrates: 84% with turtle, 84% zebra finch, 70% mice, and 77% newt.

The mRNA expression levels of Ad4BP/SF-1 were significantly higher in the gonads of male hatchlings than in those of female, while the expression of P450arom was higher in those of female than male. No sex difference was observed in the expression levels of P450scc. In the embryonic gonads, no significant effect of incubation temperature on the expression of these three genes was detected in all stages examined.

In summary, we identified the cDNA of the leopard gecko Ad4BP/SF-1 and conducted the expression analysis in the developing gonads. The male specific expression of this gene was detected in the gonads of hatchling, when morphological sex differentiation was started to be observed. This male specific expression was inconsistent with the expression pattern of two steroidogenic enzymes examined in this species. Thus, it is suggested that leopard gecko Ad4BP/SF-1 plays different role from the regulation of the expression of P450arom or P450scc in male sexual differentiation of the gonad.

P2

EXPRESSION ANALYSIS OF BDNF IN THE VENTROMEDIAL HYPOTHALAMIC NUCLEUS OF THE CHICK EMBRYO

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Though brain sexual differentiation has been thought to be regulated by the gonadal steroid hormones after sex determination of gonads, the direct effect of sex determining factor on the brain was suggested in some species. The female leopard gecko, a lizard with temperature-dependent sex determination, from different incubation temperature showed different sexual behavior such as aggressiveness and attractiveness to male. In this species, it has been reported that the mRNA expression of steroidogenic enzymes (P450scc and P450arom) were affected by incubation temperature. Because estrogen synthetase, P450arom expression was not observed in the gonads before hatching, this result strongly suggests that the brain can differentiate independently from the gonadal steroid. In this study, we will demonstrate that the sex difference of a gene expression level in the brain of the chicken, amniotic species with genotypic sex determination.

In the chick brain, the expression of cholesterol side chain cleavage enzyme (P450scc), P450arom and sex steroid hormone receptors was observed by RT-PCR from days 4.5 of incubation without sexual difference. Ad4BP/SF-1, a transcription factor of steroidogenic enzymes was also expressed in the brain from days 4.5 of incubation, however its expression at days 5.5 of incubation was significantly higher in the male brains than in the female brains. This expression was exclusively localized in the ventromedial hypothalamic nucleus (VMH). In the chick gonad, sex specific expression of estrogen synthetase (P450arom) was detected from days 6.5 of incubation. Therefore the expression of Ad4BP/SF-1 in the chick brain at days 5.5 of incubation strongly suggests the involvement of a sex steroid hormone signaling system in brain-autonomous sex differentiation in birds.

VMH is a region important for adult sexual behavior, and we analyzed brain-derived neurotropic factor (BDNF) in this brain area. BDNF has been expected as an important factor in the formation of VMH in rodents. Analysis in the chick embryonic brain by RT-PCR, its expression was detected from days 4.5, and this expression was observed in the VMH by *in situ* hybridization. This result suggests that BDNF is able to be a useful molecular marker of VMH in the chicken.

In this study, we clearly demonstrated the existence of a sex steroid hormone signaling system in the developing brain, and involvement of this system in brain-autonomous sex differentiation of both temperature-dependent and genetic-sex determination amniotes.

P3

THE EXPRESSION ANALYSIS OF DMRT1 mRNA IN THE DIFFERENTIATING TESTIS OF THE LEOPARD GECKO, SQUAMATA SPECIES WITH TEMPERATURE- DEPENDENT SEX DETERMINATION

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Dmrt1 (double sex and mab-3 related transcription factor 1) is expressed exclusively in the developing and adult testis of many vertebrates. For example, in adult mouse, like other vertebrates, the expression of *Dmrt1* was found only in the testis (2) and this expression was restricted to the Sertoli and germ cells (1). During the sexual differentiation of the mouse, *Dmrt1* was upregulated in the testis and its expression is also restricted in the Sertoli and germ cells (2). However the expression of Dmrt1 in the testis of sqamate species with temperature-dependent sex determination has not been reported. In squamate species, there are two types of sex determination, sex chromosome- and temperature-dependent sex determination even in the same family. Therefore it is important to compare the mechanism of sex determination of these species.

For this reason, we identified Dmrt1 mRNA in the leopard gecko (*Eublepharis macularius*) squamate species with temperature dependent sex determination as an experimental animal. We also examined the expression of Dmrt1 mRNA in various tissues of adults and differentiating testis of the leopard gecko.

As a result, Dmrt1 mRNA is expressed only in the testis of the adult leopard gecko, and *in situ* hybridization analysis of adult testis showed that the germ cells only expressed Dmrt1 mRNA. This is contrary to the results of other vertebrates where the Sertoli cells also expressed Dmrt1 mRNA. After thermo-sensitive period of sex determination, the expression became high in the gonads of some embryos incubated at male-biased temperature. Furthermore, just after hatching, when the testis cords begin to be visible, Dmrt1 mRNA level was high in all male gonads. These results suggest that *Dmrt1* may play a role in the differentiation of male gonads as other vertebrates.

This study suggests that in the leopard gecko, *Dmrt1* also play important role in the testis. In the future, whether Dmrt1 mRNA is expressed in the Sertoli cells during testis differentiation should be analyzed.

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P4

MIGRATION OF GnRH NEURONS IN THREE-DIMENSIONAL CULTURE OF EMBRYONIC CHICK OLFACTORY NERVE BUNDLE

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Gonadotropin-releasing hormone (GnRH), which plays a pivotal role in regulating the reproductive system, is synthesized in neurons located in the hypothalamus. In a variety of vertebrate species, GnRH neurons originate in the olfactory placode and migrate along the terminal, olfactory, and/or vomeronasal nerves into the forebrain during embryonic development (1). Vigorous researches have been performed to understand the mechanism underlying this phenomenon, uncovering factors involved in GnRH neuronal migration (2). However, many points still remain to be clarified, as the mechanism regulating the entrance of GnRH neurons to the brain or the role of GnRH secreted from those neurons. As one step to examine these issues, we established an *in vitro* model system which would be appropriate for morphological and pharmacological analysis of GnRH neuron migration.

A piece of olfactory nerve bundle (ON) along which GnRH neurons migrate was obtained from embryonic days 7.5-8 of chick, and embedded in a mixture of collagen gel and Matrigel. The ON explants were maintained for up to 5 days. After 2-3 days *in vitro*, GnRH immunoreactive (-ir) cells migrated out of the ON explants into the gel matrix. These GnRH-ir cells often contacted with each other forming cord-like structure, while some migrated independently along either GnRH-ir or non-GnRH-ir fibers. In addition, GnRH-ir neurites were observed to extend from the explants. Morphological characteristics of migrating GnRH-ir cells in this system resembled that of *in vivo*, thus this explant culture system will allow direct examination and manipulation of GnRH cell movement.

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P5

EXPRESSION PATTERNS OF AXON GUIDANCE MOLECULES AND THEIR RECEPTORS DURING GnRH NEURONAL MIGRATION FROM NOSE TO THE BASAL FOREBRIAN

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GnRH (gonadotropin-releasing hormone or luteinizing hormone-releasing hormone; LHRH) neurons originate in the olfactory placode and migrate into the forebrain during development. Examination of the normal development of GnRH neurons indicates that two major pathways of GnRH neuronal migration exist within the brain of chick embryos. The main pathway of GnRH neuronal migration largely corresponds to the course of a subset of the olfactory fibers. At the most caudal level of this pathway, many of them detach from these fibers and migrate ventrally, suggesting that the migration mode taken by these GnRH neurons is a kind of axon-independent manner (1). Attractive or repulsive chemical cues are implicated in regulating the ventral-directed migration of GnRH neurons.

Different molecules with chemotrophic functions and their receptors have been identified to play a role in axon guidance and cell migration during development of the nervous system. In this study, we have performed in situ hybridization analysis of the expression patterns of netrin-1, semaphorin (sema) 3A, slit-1 and their receptors in an attempt to identify guidance substrates that GnRH neurons guide to their final destinations. Netrin-1 expression was seen in the ventral hypothalamus, whereas was absent in the nasal region and the most rostral forebrain. A few GnRH neurons expressed netrin-1 receptor neogenin. Slit-1 was expressed in the forebrain, but its receptor robo-1 was not expressed in GnRH neurons. Sema3A expression was detected in the olfactory-forebrain region, but was absent in the medial forebrain surface where GnRH neurons migrated in association with a subset of olfactory fibers. At the caudal end of this pathway, sema3A was observed in the more dorsal region at which GnRH neurons changed their course ventrally. Many GnRH neurons expressed sema3A receptor neuropilin-1, suggesting that sema3A may act as a repellent during the migration of GnRH neurons.

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P7

SHORT– AND LONG-TERM SYNAPTIC PLASTICITY IN AREA CA1 OF THE HIPPOCAMPUS IN ADULT *rdw/rdw* RATS WITH CONGENITAL HYPOTHYROIDISM

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Although various behavioral deficits have been described in hypothyroid animals, rodent hypothyroidism models have shown impaired spatial learning and memory (1-2), in addition to deficiency in attention and hyperactivity. However, molecular mechanisms of this cognitive dysfunction have not been well studied. On the other hands, it is well known that hippocampal circuits are crucially involved in cognitive functions, such as learning and memory, in humans and other animals and express a very high degree of functional and structural plasticity. Thus, we investigated short and long synaptic plasticity including long-term potentiation (LTP) and long-term depression (LTD), paired pulse facilitation (PPF) in area CA1 of the hippocampus slice in adult *rdw/rdw* rats. The *rdw* rat shows hypothyroidism due to a missense mutation in thyroglobulin and secondary dwarfism (3).

At 60 min after high- frequency stimulation, the LTP of field excitatory postsynaptic potentials slope (S-fEPSPs) was significantly reduced to 107.3 ± 4.5 % in the hippocampus from adult hypothyroid *rdw* rats, as compared to 151.6 ± 38.3 % in that from adult normal rats, although the S-fEPSPs at 0 min were not significantly different between normal and *rdw* rats. The LTD of the S-fEPSPs 40 min after low-frequency stimulation was 92.3 ± 3.4% and 86.2 ± 3.6% in *rdw* hypothyroid and normal rats, respectively. There was no significant difference between *rdw* and normal rats. The paired-pulse ratios were recorded at various interstimulus intervals ranging from 30 to 950 ms, significantly lowered mean PPF at interstimulus intervals between 120 and 200 ms in *rdw* rats as compared to that from normal rats. This result suggests that presynaptic glutamate releases were increased by *rdw* rats.

Reduction of LTP and PPF ratio observed in *rdw* hypothyroid rats in this work implicates that cognitive dysfunction including learning , memory, attention and hyperactivity, may occur in *rdw* hypothyroid rats.

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P8

PACAP INDUCES DIFFERENTIATION OF MOUSE EMBRIONIC NEURAL STEM CELLS INTO ASTROCYTES *IN VITRO* AND *IN VIVO*

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Pituitary adenylate cyclase-activating polypeptide (PACAP) is a neuropeptide that exerts a wide range of effects on different cell types in brain as early as fetal developmental stage. Distribution and localization of PACAP and its specific receptor (PAC1-R) mRNA during development suggests that PACAP is associated with proliferation and differentiation of neural stem cells (NSCs) (1). However, few is known on the functional role of PACAP in NSCs.

We have already shown that PACAP induces differentiation of cultured mouse embryonic NSCs into astrocytes. When NSCs were exposed to 2 nM PACAP instead of bFGF for 8 days, 55% of these cells were immunostained with anti-glial fibrally acidic protein (GFAP) antibody. This phenomenon was completely diminished with $PACAP_{6-38}$, PAC1-R antagonist treatment (2). The signal transduction pathway of PACAP signaling was further investigated in detail. This differentiation was mimicked by protein kinase C (PKC) activator (PMA) but suppressed by phospholipase C inhibitor (U73122), intracellular calcium chelator (BAPTA-AM), and selective inhibitor of PKC (chelerythrine). Furthermore, overexpression of PKCβII with adenovirus vector synergistically enhanced differentiation in the presence of 1 nM PACAP, whereas expression of the dominant-negative mutant of PKCβII proved inhibitory, suggesting that the β isoform of PKC plays a crucial role in the PACAP-induced NSCs differentiation (3).

Functional significance of PACAP on astroglial differentiation *in vivo* was studied with immunohistochemical analysis at E14, 16, 18, P1 and P3 in mouse brain. PAC1-R was co-localized with nestin in the neural progenitor cells of E14 mouse ventricular zone. GFAP immunopositive cells appeared in the region where PAC1-R was strongly expressed. These results strongly suggest that PACAP play very important roles in astroglial differentiation from NSCs during brain development.

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P9

EFFECT OF PITUITARY ADENYLATE-CYCLASE ACTIVATING POLYPEPTIDES (PACAP) ON ADULT NEURAL STEM CELLS

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Mammalian adult brain was thought to be completely incapable of neurogenesis, the birth of new neuron. In the last decade, it was defined that neurogenesis normally occurred in specific region, existed neural stem cells (NSCs), of the adult mammalian brain. Recent findings show that it is possible to induce the neurogenesis, and the generated neuron may replace lost or damaged neuron. However, the regulatory systems of neurogenesis are not well understood. Pituitary adenylate cyclase-activating polypeptide (PACAP) is a pleiotropic neuropeptide, which was mainly distributed in the hypothalamus, and the specific receptor (PAC1R) was widely expressed in the brain (1, 2). It has been reported that PACAP stimulates a proliferation or differentiation of NSCs derived from fetal brain *in vitro*. However, effects of PACAP on neurogenesis in adult mouse brain are poorly known. Therefore, we first observed the distribution of PAC1R immunoreactivity in the two neurogenic region, subventricular zone (SVZ) and subgranular layer (SGL） of dentate gyrus of hippocampus. PAC1R immunoreactivity was co-localized with NSC marker, nestin in SVZ, but not with neuroblast marker, doublecortin, suggesting PACAP affects NSCs (3). Subsequently, to make clear the effect of PACAP on adult NSCs, thymidine analog, BrdU was intraperitoneally injected to label a proliferating nucleus following intraventricularly infusion of PACAP for 1 week. The number of BrdU immunopositive cells in the SGL of dentate gyrus of hippocampus was significantly increased in PACAP injected mouse brain rather than vehicle injected group. Moreover, neurogenesis induced by ischemic model, common carotid artery occlusion (CCAO) was compared with wild type and PACAP heterogeneous knockout (PACAP+/-KO) mice by BrdU labeling. BrdU immunopositive cells in SGL increased at 3 to 7days after CCAO; however the number of the cells did not change in PACAP+/-KO mice after ischemia. These results suggest that PACAP is a key factor for proliferation of NSCs in mouse brain.

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P10

EXPRESSION OF RETINALDEHYDE DEHYDROGENASE (RALDH) mRNA IN THE CHICKEN EMBRYONIC PITUITARY GLAND

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Retinoic acid (RA), the active derivative of vitamin A, is known to be an important regulator of essential aspects of organogenesis. Previous studies on the temporal and spatial expression patterns of retinaldehyde dehydrogenase (RALDH) mRNA in the pituitary gland (1,2) and effects on hormone expression in a cultured cell line have suggested that RA plays an important role in pituitary development and differentiation of hormone-producing cells. However, a direct role of RA in development of the pituitary gland remains obscure. This issue must be investigated by using chicken embryos because chicken embryos can develop independently from maternal vitamin A and can be easily cultured. In this study, we investigated the spatial and temporal expression patterns of RALDH2 or 3 mRNA in embryonic chicken anterior pituitary glands using RT-PCR and *in situ* hybridization. RALDH3 mRNA-expressing cells were observed in the primordium of the anterior pituitary gland from embryonic day 3 (E3), and the expression of RALDH3 mRNA was consistently strong in the dorsal part of the gland close to the base of the diencephalon and was weak in the ventro-caudal part of the pituitary gland. Interstingly, the intensity of RALDH3 mRNA expression in the anterior pituitary gland gradually decreased after E12 and then strong RALDH3 mRNA expression appeared in the pars tuberalis. To identify the hormone-producing cells that express RALDH3 mRNA, we performed double staining for RALDH3 mRNA and anterior pituitary hormones, and we found that POMC-, αGSU-, LHβ-, FSHβ-and TSHβ-producing cells were not overlapped with RALDH3 mRNA-expressing cells. Moreover, RALDH2 mRNA expressing-cells were found in the primordium of the pars tuberalis at E8. These results suggest that RA is generated by RALDH2 and RALDH3 in the chicken embryonic anterior pituitary gland and that RA may be closely associated with the pars tuberalis.

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P11

CHANGES IN EXPRESSION OF GASTRIC GHRELIN, AROMATASE AND LEPTIN IN THE POSTNATAL RAT STOMACH

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Ghrelin, a 28-amino-acid peptide isolated from the rat stomach as an endogenous ligand for growth hormone secretagogue receptor, has been shown to stimulate GH release, food intake and gastric acid secretion. In an adult rat, it has been reported that gastric ghrelin expression and release are regulated by estrogen, somatostatin, leptin and insulin of the stomach. However, the relationships between gastric ghrelin and these regulatory factors in postnatal development remain obscure. In this study, we investigated the changes in gastric ghrelin and these factors at each postnatal stage by real-time quantitative PCR and double staining for ghrelin-producing cells and aromatase- or leptin- expressing cells.

Postnatal ghrelin mRNA expression level significantly increased between 2 weeks (2 wk) and 4 wk and between 4 wk and 8 wk in male and female rats respectively. The expression levels of gastric estrogen synthases (aromatase, P450 17α, 17β HSD typeⅢ) also significantly increased between 2 wk and 4 wk in male and female rats. On the other hand, expression level of gastric leptin mRNA level decreased between 2 wk and 4 wk. Double staining for expression of aromatase or leptin mRNA by *in situ* hybridization and ghrelin-producing cells by immunohistochemistry revealed that these cells were located near each other, and ghrelin-producing cells were usually found adjacent to leptin- and aromatase-expressing cells in the gastric mucosa through the period of postnatal development.

The results suggest that the gradual increase in gastric ghrelin expression level in the postnatal rat is caused by the inverse changes in production of gastric leptin and estrogen and that release of these factors regulate ghrelin expression in a paracrine manner.

P12

mRNA EXPRESSION PATTERNS OF IGFBPS IN DEVELOPING LIMB

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Insulin-like growth factor (IGF) is mainly secreted from liver and partly by an autocrine or paracrine fashion. IGF is important for both fetal and postnatal development. In addition, it also controls tissue homeostasis via regulation of cell proliferation and apoptosis. There are several types of IGF-specific binding protein, insulin-like growth factor binding proteins (IGFBPs). They have great affinity to IGF and render the IGF system much more complex. However, the mechanism of IGF-IGFBPs axis has not been fully elucidated. The formation of the digits in amniota embryos is accomplished by the proliferation of the digital segment and the apoptotic cell death of the interdigital zone simulutaneously. We focused on the formation of the limb and investigated the IGFBPs expression in that of Japanese quail. In this study, by whole mount *in situ* hybridization, IGFBP2 and IGFBP4 were detected in the outgrowth region and in apoptotic zone respectively. These results indicate that IGFBPs are important for characterizing the IGF function in avian morphogenesis. Then, we treated the embryos with BrdU which inhibit apoptosis in interdigital zone. The treated quail hatched with webbed-limb. Though the inhibition of the apoptosis was observed in interdigital zone, there were no differences in expressions of IGFBPs. Therefore, the inhibiting apoptosis by the BrdU might not be concerned with IGFBP. Hence, we are planning to compare the IGFBPs expressions of the quail with that of waterfowl which has webbed-limb.

P13

EFFECT OF ESTROGEN AND PROLACTIN ON THE PERCEPTION OF SODEFRIN IN FEMALE NEWTS, *Cynops pyrrhogaster*

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Sodefrin, a female-attracting pheromone of the newt *Cynops pyrrhogaster*, has been presumed to be perceived by the sensory neurons of the lateral nasal sinus (LNS) of female newts (1). LNS is a diverticulum situated lateral part of the main chamber of the nasal cavity, which has been considered to correspond to the vomeronasal organ (VNO) in other quadrupeds (2), where pheromones are mainly detected.. In VNO of rodents, intracellular Ca^{2+} ($[Ca^{2+}]_i$) levels are known to be elevated by the stimulations with urinary extract containing potential pheromones (3). As a step to study the mechanisms of sodefrin perception, measurement of $[Ca^{2+}]_i$ in the dispersed LNS cells following the exposure to sodefrin by microfluorometry with fura-2 AM was carried out. Increase of $[Ca^{2+}]_i$ was detected in a small number of LNS cells from sexually developed female newts after the application of sodefrin ($>10^{-10}$ M), whereas sodefrin-responsive cells were very rarely detected in the males or sexually undeveloped females. This coincides well with our previous observation that responsiveness of LNS cells to sodefrin as detected by electro-olfactogram is sex-dependent and hormone (estrogen and prolactin)-dependent. In fact, it was confirmed that LNS cells were equipped with both estrogen and prolactin receptors. In ovariectomized and hypophysectomized sexually developed females, sodefrin-responsive cells, as detected by Ca^{2+} imaging method, decreased in number. This decrease was restored by the supplementation with estradiol and prolactin. It has also been known that treatment of female newts with estradiol and prolactin elicits proliferation of LNS cells. Thus, it was concluded that in female newts, this combination of hormones enhances the responsiveness to sodefrin and increases the population of LNS cells.

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P14

ISOLATION OF MOLECULES WHICH INTERACT WITH HSP90b IN GERM CELL APOPTOSIS IN NEWT TESTIS

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We have shown in vivo and in vitro that prolactin induces apoptosis in the 7th generation of spermatogonia during newt spermatogenesis, but the underlying molecular mechanism remained unknown. Western blotting has detected Tyrosine phosphorylation of heat shock protein (HSP) 90β, a molecular chaperone for client proteins in signal transduction and transcriptional regulation, by prolactin in newt testis, and co-immunoprecipitation has demonstrated that HSP90β associated constitutively with prolactin receptor on the plasma membrane of germ cells.

To determine the role of HSP90β in prolactin-induced apoptosis, we performed co-immunoprecipitation with an antibody against C terminal region of nHSP90β that we produced. HSP90β was demonstrated to associate with some proteins and undergo constitutive phosphorylation in newt testis. Furthermore, injection of newts with prolactin caused dissociation of HSP90β from prolactin receptor, while injection with follicle-stimulating hormone (FSH) caused dephosphorylation of a protein (approx. 38 kDa of the molecular weight) associated with HSP90β. Taken together, these results suggest that HSP90β is involved in signaling prolactin-induced apoptosis through the receptor and in signaling FSH-induced initiation of meiosis. In future, we will identify the proteins associated with HSP90β by proteomics, and then examine their expressions in newt testis.

P15

ABSENCE OF GENITALIN (GONAD-STIMULATING HORMONE)-INDUCED 1-METHYLADENINE PRODUCTION IN OVARIAN FOLLICLE AND TESTICULAR INTERSTITIAL CELLS OF STARFISH GONADS DURING GAMETOGENESIS

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Recently, we and collaborators purified and identified the gonad-stimulating hormone in starfish, *Asterina pectinifera* (1). The starfish gonadotropic hormone was a dimeric peptide like insulin. Here, we propose the hormone to be named "genitalin" as a novel gonad-stimulating insulin-like neuropeptide hormone, instead of "gonad-stimulating substance (GSS)". To obtain further information about hormonal action of genitalin, this study examined effect of the synthetic genitalin on 1-methyladenine (1-MeAde) production in ovarian follicle and testicular interstitial cells obtained from growing and ripe gonads of starfish *A. pectinifera*. The synthetic genitalin stimulated ripe follicle cells as well as ripe interstitial cells to produce 1-MeAde through arise in cyclic AMP (cAMP). However, neither 1-MeAde nor cAMP production occurred in follicle and interstitial cells at the growing stage under the presence of genitalin. According to competitive experiments (2) using radioiodinated and radioinert genitalin in ovarian follicle cells, *Kd* value in ripe stage was lower than that in growing stage, though almost the same Bmax value was obtained in both stages. The result suggests that the affinity of receptors for genitalin in ovarian follicle cells becomes high enough to activate adenylyl cyclase in the breeding season. In contrast, *Kd* and Bmax values in interstitial cells at the growing stage were almost the same as those at the ripe stage. It is unclear why genitalin is failed to produce 1-MeAde and cAMP in interstitial cells at the growing stage. It may be possible that G-protein or adenylyl cyclase in these cells is not coupled with the genitalin receptors.

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P16

cDNA CLONING, GENE STRUCTURE, AND EXPRESSION OF KISS1 AND GPR54 IN MEDAKA

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KISS1 was initially identified as a metastasis suppressor gene. Recent studies have demonstrated that the KISS1 products metastin/kisspeptin are endogenous agonists for a G protein-coupled receptor, GPR54. In mammals, the KISS1-GPR54 system is a key regulator of the reproductive system that regulates the hypothalamic gonadotrophin-releasing hormone. However, the roles of the KISS1-GPR54 system in nonmammalian vertebrates remain to be elucidated.

To obtain molecular basis on the roles of the KISS1-GPR54 system in teleost, in the present study, we cloned cDNA encoding KISS1 and GPR54 in medaka *Oryzias latipes* and their gene structures were clarified. In addition, effects of photoperiod on the expression of KISS1 and GPR54 genes were examined.

The medaka KISS1 cDNA (759 bp) is encoding 100 amino acid residues. The medaka KISS1 gene is composed of three exons and two introns. The coding region of the medaka GPR54 cDNA (1131 bp) is encoding 376 amino acid residues. The GPR54 gene is composed of five exons and four introns.

RT-PCR analyses demonstrated the expression of KISS1 and GPR54 in all tissues examined (brain, retina, gonads, and so on). Quantitative real-time PCR analyses demonstrated that there is no difference in the amount of KISS1 and GPR54 transcripts between the whole brain samples collected under long day and short day both in females and males.

Further studies should be required to elucidate the roles of the KISS1-GPR54 system in this species.

P17

GnIH NEURONS PROJECT TO GnRH NEURONS IN THE AVIAN BRAIN

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The decapeptide gonadotropin-releasing hormone (GnRH) is the primary factor responsible for the hypothalamic control of gonadotropin secretion. Until recently, no neuropeptide acting at the level of the pituitary to regulate gonadotropin secretion negatively had been identified in vertebrates, although gonadal sex steroids and inhibin inhibit gonadotropin secretion *via* feedback from the gonads. In 2000, we discovered a novel hypothalamic dodecapeptide which acts on the pituitary to inhibit gonadotropin release in the Japanese quail and termed it gonadotropin-inhibitory hormone (GnIH; 1). The action of GnIH is mediated by a novel G protein-coupled receptor (2). This gonadotropin-inhibitory system may be widespread in vertebrates, at least birds and mammals (3-6). In these higher vertebrates, histological evidences suggest contact of GnIH immunoreactive axon terminals with gonadotropin-releasing hormone (GnRH) neurons (6, 7), thus indicating direct regulation of GnRH neuronal activity by GnIH. In this study we investigated the interaction of GnIH and GnRH-I and -II neurons in European starling brain. Cloned starling GnIH precursor cDNA encoded three peptides which possess characteristic LPXRF-amide (X = L or Q) motifs at the C-termini. Starling GnIH was further identified as SIKPFANLPLRF-NH_2 by mass spectrometry combined with immunoaffinity purification. GnIH neurons, identified by *in situ* hybridization (ISH) and immunocytochemistry (ICC), were clustered in the hypothalamic paraventricular nucleus. GnIH immunoreactive fiber terminals were present in the external layer of the median eminence in addition to the preoptic area and midbrain, where GnRH-I and GnRH-II neuronal cell bodies exist, respectively. GnIH axon terminals on GnRH-I and -II neurons were shown by GnIH and GnRH double-label ICC. Further, the expression of starling GnIH receptor mRNA was identified in both GnRH-I and GnRH-II neurons by ISH combined with GnRH ICC. Thus GnIH may regulate reproduction of vertebrates by directly modulating GnRH-I and GnRH-II neuronal activity, in addition to influencing the pituitary gland.

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P18

GONADOTROPIN-INHIBITORY HORMONE AND ITS RECEPTOR IN THE AVIAN REPRODUCTIVE SYSTEM

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We recently identified a novel hypothalamic dodecapeptide which acts on the pituitary to inhibit gonadotropin release in the quail and termed it gonadotropin-inhibitory hormone (GnIH; 1). GnIH inhibits gonadal development and maintenance by decreasing gonadotropin synthesis and release in birds (1-7). Many hormones that are classified as neuropeptides are synthesized in vertebrate gonads in addition to the brain. Receptors for these hormones are also expressed in gonadal tissue; thus there is potential for a highly localized autocrine or paracrine effect of these hormones on a variety of gonadal functions. In the present study, we therefore investigated whether GnIH and its receptor are expressed in the avian reproductive system including gonads and accessory reproductive organs by studies on two orders of birds: Passeriformes and Galliformes. Binding sites for GnIH were initially identified *via in vivo* and *in vitro* receptor fluorography, and were localized in ovarian granulosa cells along with the interstitial layer and seminiferous tubules of the testis. Furthermore, species-specific primers produced clear PCR products of GnIH and GnIH receptor (GnIH-R) in songbird and quail gonadal and other reproductive tissues, such as oviduct, epididymis and vas deferens. Sequencing of the PCR products confirmed their identities. Immunocytochemistry detected GnIH peptide in ovarian thecal and granulosa cells, testicular interstitial cells and germ cells and pseudostratified columnar epithelial cells in the epididymis. *In situ* hybridization of GnIH-R mRNA in testes produced a strong reaction product which was localized to the germ cells and interstitium. In the epididymis, the product was also localized in the pseudostratified columnar epithelial cells. In sum, these results indicate that the avian reproductive system has the capability to synthesize and bind GnIH in several tissues. The distribution of GnIH and its receptor suggest a potential for autocrine/paracrine regulation of gonadal steroid production and germ cell differentiation and maturation.

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P20

IN VIVO ACTION OF OCTOPUS GNRH: PHARMACOLOGICAL EFFECTS AND IMMUNOCYTOCHEMISTRY IN HATCHLING BRAINS

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Previous studies suggest that oct-GnRH acts not only as a hormone regulating gonadal maturation of octopus, but also as a multifunctional modulatory factor in the central and autonomic nervous systems (1). To understand in vivo effects, we injected the peptide into the optic blood sinus of hatchlings (*Octopus bimaculoides*), and their behaviors were observed. Action of oct-GnRH caused several abnormalities such as unstable crawl, postures, abnormal movements of arms, hyperactivities of mantle, hearts, and chromatophores, and ejection of yolk. These effects continued at least 1 hour, then these specimens recovered the normal behavior 1 day after.

Oct-GnRH-like immunoreactive neural cell bodies were distributed in the anterior subesophageal mass (movement of arms), posterior subesophageal mass (chromatophore, visceral, and funnel movements), optic tract lobe and basal lobe (posture control) of the brain. Immunoreactive fibers were distributed in neuropils of these mass and lobes. Effects of oct-GnRH on behaviors can be interpreted as pharmacological activities on these immunoreactive neurons and fibers. Yolk is considered to be a part of content in digestive organs of hatchlings. So that, ejection of yolk may be attributed to intestinal contractions caused by oct-GnRH. Peripherally, oct-GnRH exhibited contractile activities on the small intestine and rectum. Furthermore, the immunoreactivites were also observed in the olfactory lobe and superior buccal lobe (higher center of taste and touch), inferior frontal lobe (higher center of touch), superior frontal lobe (higher center of vision), and vertical lobe (higher center of vision and touch), suggesting that oct-GnRH may have important roles for higher brain functions as a neurotransmitter and/or neuromodulator.

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P21

DES INDUCES FISH OOCYTE MATURATION THROUGH MEMBRANE PROGESTIN RECEPTOR

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The potency of endocrine-disrupting chemicals (EDCs) to induce or prevent the resumption of meiotic cell cycle by maturation-inducing steroid (MIS) was examined *in vitro* oocyte culture. We found that exposing fish oocytes to diethylstilbestrol (DES) at a dose within a range similar to that used in experimental exposure to a natural MIS: 17α, 20β-dihydroxy-4-pregnen-3-one (17, 20β-DHP) (1). The result was the first report showing that EDC can potentially induce oocyte maturation like an endogenous MIS. Maturation inducing activity was also evaluated by the detection of synthesis of cyclin B protein. A synergistic action of DES on 17, 20β-DHP-induced oocyte maturation was observed. These results suggested that DES acted on MIS receptor.

Recently a membrane bound progestin receptor, named as membrane progestin receptor (mPR), was identified as a strong candidate for MIS receptor. To investigate possible interaction between mPR and DES, cDNAs for mPR proteins were isolated from goldfish ovarian cDNA library (2). The specific antibody for goldfish mPRα was raised against recombinant mPRα expressed in *E.coli*. Both 17, 20β-DHP- and DES-induced oocyte maturation was inhibited by an antibody against mPRα. The ability of DES and its analogues to interact with the progestin receptor mediating oocyte maturation was investigated in receptor binding assays using plasma membranes from goldfish ovaries and breast cancer cells transfected with goldfish mPRα. Membranes prepared from both ovaries and mPRα–transfected cells showed high affinity, saturable, displaceable, single binding sites specific for the 17, 20β-DHP. DES and DES analogues (DP-DES and HEX), which induce oocyte maturation in goldfish, bound to the receptor and caused concentration-dependent displacement of [^{3}H]-17, 20β-DHP (3).

The relative binding affinities of various steroids and EDCs to these two receptor preparations were similar, supporting the idea that mPRα is a MIS receptor *in vivo*. The close correspondence between binding of DES and its analogues to the mPRα protein and their oocyte maturation-inducing activities suggests a mechanism of endocrine disruption mediated by binding to mPRα, thereby mimicking the nongenomic action of the 17, 20β-DHP.

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P22

ISOLATION AND CHARACTERIZATION OF cDNA ENCODING NEUROMEDIN U (NMU) RECEPTOR FROM THE BRAIN OF GOLDFISH

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In rodents, neuromedin U (NMU) is a multifunctional neuropeptide implicated in the regulation of the circulatory and digestive systems and energy homeostasis, especially appetite (1). However, there is no available information on the nature and physiological roles of NMU and its receptor in fish. In the present study, we identified four cDNAs encoding three NMU orthologs from the goldfish brain and gut. Putative peptides consisting of 21, 25 and 38 amino acid residues (NMU-21, -25 and -38) were deduced from their nucleotide sequences. In the brain, mRNA for NMU-21 was strongly expressed. Intracerebroventricular (ICV)-injected synthetic NMU-21 suppressed food intake in a dose-dependent manner. We also isolated two cDNAs encoding NMU receptor orthologs (NMU-R1 and NMU-R2) from the brain. The amino acid sequences of goldfish NMU-R1 and -R2 have 49% and 43% homology between that of human NMU-R1 and -R2, respectively. These mRNAs were expressed in the brain, pituitary, gill, kidney, testis and ovary while in the gut, NMU-R2 mRNA expressed strongly. These results suggest that NMU orthologs and their receptors exist in fish, and that the NMU-21 thus deduced can potently inhibit food intake in goldfish (2).

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P23

PURIFICATION AND PROPERTIES OF GHRELIN FROM THE INTESTINE OF GOLDFISH, *Carassius auratus*

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Ghrelin is a 28-amino acid peptide identified in rat and human stomach as an endogenous ligand for the growth hormone secretagogue receptor (GHS-R) [1]. Ghrelin is now recognized to be a multifunctional peptide that is involved in the regulation of somatic growth, feeding behavior and energy homeostasis in mammals. In goldfish, cDNA encoding the ghrelin precursor has been identified [2]. Ghrelin mRNA is mainly expressed in the intestine (given that goldfish lack a stomach), and few in the brain. We have indicated that, in the goldfish intracerebroventricular (ICV) and intraperitoneal administrations of synthetic goldfish ghrelin (12 amino acid-residues form with an octanoic acid modification at the third N-terminal serine residue (Ser^3), 12C8) stimulate food intake and locomotor activity [3]. However, the native mature ghrelin generated from the ghrelin precursor has not yet been identified in goldfish. In the present study, we purified and characterized ghrelin from the intestine of goldfish. Ghrelin thus obtained from the intestine has 11 molecular forms consisting of 14, 17, 18 and 19 amino acid-residues. Ser^3 was acylated by *n*-octanoic, *n*-nonanoic or *n*-decanoic acid. Since the 17 amino acid-residues form was much yielded, we synthesized 17 amino acid-residues with or without the octanoic or decanoic acid modification at Ser^3 (17C0, 17C8 and 17C10), and examined their biological activity. 17C8 and 17C10, but not 17C0, increased intracellular calcium concentrations in a cell line stably expressing rat GHS-R with similar potency to 12C8. ICV administration of 17C8, but not 17C0 and 17C10, increases food intake as well as 12C8 in the goldfish.

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EFFECT OF GOLDFISH LPXRF-AMIDE PEPTIDES ON FOOD INTAKE IN GOLDFISH, *Carassius auratus*

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Novel neuropeptides, which are characterized by a similar structure including the C-terminal LPXRF-NH_2 motif (X = L or Q residue), have been isolated from tetrapods (1). In the goldfish brain, a cDNA encoding the precursor of three LPXRF-NH_2 peptides (gfLPXRFa-1, -2 and -3) has also been identified (2). Their mRNA is specifically expressed in the diencephalon, and neuronal cell bodies expressing the precursor are located in the hypothalamus, the nucleus posterioris periventricularis (NPPv). The NPPv is one of centers implicated in the regulation of feeding behavior in goldfish, however it is unknown whether gfLPXRFa-1, -2 and -3 affect food intake. In the present study, we examined the effect of intracerebroventricular (ICV) administration of gfLPXRFa-1, -2 and -3 on food consumption in goldfish. ICV injection of graded doses of gfLPXRFa-2 (0.1-10 pmol/g BW) induced a dose-dependent inhibition of food intake, a significant decrease in cumulative food intake during the 60-min period after feeding being observed at doses of 1 and 10 pmol/g BW. On the other hand, ICV-injected gfLPXRFa-1 and -3 at same doses have no evident changes in food intake. These results suggest that gfLPXRFa-2 has a potency to act as an anorexigenic factor involved in the feeding regulation of goldfish.

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INHIBITORY EFFECT OF MELANIN-CONCENTRATING HORMONE ON FOOD INTAKE IN GOLDFISH

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Melanin-concentrating hormone (MCH) was originally isolated from the chum salmon pituitary as a hormone involved in body color regulation (1). Recently, it has been indicated that MCH is implicated in regulation of feeding behavior and energy homeostasis in mammals (2). However, the role of MCH in appetite has not yet been well studied in fish. We have indicated that MCH influences feeding behavior in the goldfish, and exerts an anorexigenic action, unlike its orexigenic effect in mammals (3, 4). This raises the question of why MCH acts as an anorexigenic factor in goldfish. Therefore, the aim of the present study was to investigate the involvement of anorexigenic or orexigenic neuropeptides in the anorexigenic action of MCH in goldfish. Because α-melanocyte-stimulating hormone (α-MSH) and corticotropin-releasing hormone (CRH) are both powerful and key anorexigenic neuropeptides in goldfish, we examined the effects of intracerebroventricular (ICV) injection of an α-MSH receptor (melanocortin 4 (MC4) receptor) antagonist, HS024, and a CRH 1/2 receptor antagonist, α-helical $CRH_{(9\text{-}41)}$, on the anorexigenic action of ICV-administered MCH. ICV injection of HS024, but not α-helical $CRH_{(9\text{-}41)}$, suppressed MCH-induced anorexigenic action for a 60 min observation period. We also examined, using a real-time PCR method, whether ICV-injected MCH affects the expression of orexigenic neuropeptide mRNAs, such as neuropeptide Y (NPY), orexin, ghrelin and agouti-related peptide (AgRP), in the goldfish diencephalon. ICV administration of MCH at a dose sufficient to inhibit food consumption decreased the expression of mRNAs for NPY and ghrelin, but not for orexin and AgRP. In addition, we investigated the neuronal relationship between MCH-containing nerve fibers and α-MSH- or NPY-containing neuronal cell bodies in the hypothalamus using a double-immunofluorescence method. MCH-containing nerve fibers were located in close proximity to α-MSH- or NPY-containing neuronal cell bodies in the hypothalamus. These results indicate that the anorexigenic action of MCH in the goldfish brain is mediated by α-MSH signaling pathway, perhaps through the MC4 receptor system, with inhibition of hypothalamic NPY and ghrelin synthesis.

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P26

DIAZEPAM-BINDING INHIBITOR-DERIVED PEPTIDES-INDUCED ANOREXIGENIC ACTION IS MEDIATED THROUGH THE METABOTROPIC ENDOZEPINE RECEPTOR IN GOLDFISH

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An endogenous ligand of central-type benzodiazepine receptors (CBR), the endozepine octadecaneuropeptide (ODN), is a very potent inhibitor of food intake in rodents (1-3). Although endozepines have been localized and characterized in the trout hypothalamus, so far, the action of these neuropeptides on feeding behavior has never been investigated in fish. In the present study, we have examined the effect of ICV administration of synthetic rat ODN, its C-terminal octapeptide (OP) and the head-to-tail cyclic analog $cyclo_{1-8}$OP (cOP) on feeding behavior in the goldfish model. ICV injection of graded doses of ODN (2.5-10 pmol/g BW) induced a dose-dependent inhibition of food intake, a significant decrease in cumulative food intake during the 60-min period after feeding being observed at doses of 5 and 10 pmol/g BW. The inhibitory effect of a 10 pmol/g BW dose of ODN on food consumption (-39%) was mimicked by an equimolar dose of OP (-42%) and cOP (-53%). The food intake-suppressing activity of ODN (10 pmol/g BW) was not affected by pre-injection of the CBR antagonist flumazenil (200 pmol/g BW). In contrast, the anorexigenic effect of ODN (10 pmol/g BW) was totally suppressed by a selective antagonist of metabotropic endozepine receptors, $cyclo_{1-8}$[DLeu^5]OP. These data indicate that, in goldfish as in rodents, ODN is a potent inhibitor of food consumption, and that the anorexigenic effect of ODN is not mediated through CBR but through the metabotropic endozepine receptor (4).

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P27

DISTRIBUTION OF MELANOCORTIN RECEPTORS AND EFFECTS OF FEEDING ON ITS EXPRESSION WITHIN BARFIN FLOUNDER, PLEURONECTIFORMES

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Melanocyte-stimulating hormone (MSH) and adrenocorticotropic hormone (ACTH) are collectively termed melanocortin (MC). These peptides are derived from a precursor protein, proopiomelanocortin (POMC). MSH and ACTH participate in body color changes and stress responses, respectively. The functions of MC are mediated through the MC receptor (MCR) family, which are G protein-coupled receptors with seven transmembrane domains, showing a specific tissue distribution and pharmacological properties. Five MCR subtypes have been identified in mammals and six and four in pufferfish and zebrafish, respectively. The goal of our studies on MCR is the comprehensive understanding of melanocortin systems consisting of MC and MCR in fish. So far, we have identified three subtypes of POMC from barfin flounder *Verasper moseri*, Pleuronectiformes (1, 2). Herein, we report the molecular cloning, tissue distribution, and expression of MCR subtypes in barfin flounder.

We determined the entire nucleotide sequences of four MCR subtype cDNAs. The deduced amino acid sequence length is 323 for MC1R, 300 for MC2R, 325 for MC4R, and 342 for MC5R. The reverse transcription polymerase chain reaction showed a higher expression of these four subtypes in the brain and testis than in other tissues examined. Furthermore, prominent expression was also observed in the eyeball and skin for MC1R, head kidney for MC2R, eyeball, liver, and ovary for MC4R, and skin for MC5R. The diverse tissue distribution of MCRs indicates the multifunctional nature of MC systems in addition to classical functions such as melanin synthesis in skin and cortisol release in the head kidney. The lipolytic activity of MSH has been shown in the liver of rainbow trout (3). In barfin flounder liver, we found four-fold higher expression levels of MC4R in fasted than fed fish. MC seems to be associated with energy metabolism through MC4R in the liver.

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EFFECTTS OF ADVANCE OF AGE IN THE POSTNATAL PERIOD AND FASTING ON GHRELIN EXPRESSION IN THE RAT DUODENUM

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Ghrelin, an endogeneous ligand for the growth hormone secretagogue receptor, is a 28-amino-acid peptide and is produced predominantly in the stomach mucosa (1). However, after gastrectomy, nealy 30% of ghrelin remains in plasma (2), and the major source of the remaining ghrelin is thought to be the small and large intestines. Recently, several studies have shown that the gastric ghrelin mRNA expression and secretion is up-regulated by gastric estrogen (3) and down-regulated by somatostatin and leptin (4, 5). However, the regulatory mechanism of ghrelin mRNA expression level in the intestine, the most ghrelin producing region in the intestines, is still unclear. In this study, we focused on the duodenum, the most large part of ghrelin producing region in the small intestine, and studied the effect of fasting on the duodenum ghrelin expression and ghrelin mRNA expression levels at each neonatal and postnatal stage (1 week old and 2 weeks old; lactation period, 4 weeks old; puberty, 8 weeks old; maturation period) by quantitative PCR, *in situ* hybridization (ISH) and immunohistochemistry (IHC).

The quantitative RT-PCR results showed that ghrelin mRNA was expessed in the duodenum from embryonic day 18 and that the expression level increased remarkbly just after birth. Although gastric ghrelin expression level gradually increased in the postneonatal rat and then the duodenum ghrelin expression was maintained at a high level until the lactation period, the ghrelin expression level significant decreased at puberty and the maturation period. The number of ghrelin-expressing and producing-cells detected by ISH and IHC showed the same changes as those found by quantitative RT-PCR. After 48 hrs of fasting, unlike in the stomach, the duodenal ghrelin mRNA expression level was not changed. Moreover, expression levels of aromatase, leptin and somatostatin in the duodenum were not changed throughout the period of postnatal development.

The results suggested that rat duodenal ghrelin expression is controlled by mechanisms different to those that control gastric ghrelin expression.

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P29

THE OREXIGENIC EFFECT OF GONADOTROPIN-INHIBITING HORMONE IS MEDIATED BY OPIOID SYTEM IN THE BRAIN OF CHICKS

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Gonadotropin-inhibiting hormone (GnIH) is a neuropeptide which suppresses gonadotropin release from the anterior pituitary (1). Recently, we found that intracerebroventricular (ICV) injection of GnIH stimulates feeding behavior in chicks (2). However, the mechanism underlying the orexigenic effect has not been clarified. In the present study, we investigated whether the orexigenic effect of GnIH is mediated by opioid, nitric oxide or noradrenergic systems, which are well known as feeding stimulatory factors in the brain of chicks. The GnIH-induced feeding behavior was significantly attenuated by ICV co-injection of opioid mu-receptor antagonist but not delta- and kappa-antagonists, indicating that the orexigenic effect of GnIH is mediated by opioid mu-receptor. On the other hand, ICV co-injection of nitric oxide synthase inhibitor did not affect the orexigenic effect of GnIH. Adrenergic alpha2 receptor antagonist showed the tendency to attenuate the GnIH-induced feeding behavior although the significant effect was not observed. In conclusion, the present study revealed that the orexigenic effect of GnIH is related to opioid system in the brain of chicks.

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P30

MORPHOLOGICAL OBSERVATION OF NEUROPEPTIDE W NEURON NETWORK IN THE RAT HYPOTHALAMUS

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Neuropeptide W (NPW) is a novel neuropeptide which was recently isolated from the porcine hypothalamus. NPW is shown to be an endogenous ligand for GPR7 and GPR8 of the orphan G protein-coupled receptors (1, 2). GPR7 and GPR8 are expressed in brain regions, especially in the hypothalamus, which are related to feeding regulation. Intracerebroventricular (icv) infusion of NPW into the rat cerebral ventricle increases food intake for 2 h in the light phase and stimulates prolactin and corticosterone release (1). On the other hand, in the dark phase, icv infusion of NPW reduces food intake for 48 h with up-regulation of energy expenditure and decrease of body weight (3). However immunohistochemical studies on the distribution and localization of NPW have revealed differing results concerning whether or not NPW-containing cell bodies and their processes are present in the hypothalamus (4, 5). To determine the distribution of NPW in rat brain, we used specific and high-affinity antiserum against NPW to demonstrate immunohistochemically the distribution and localization of NPW-containing neurons in the rat hypothalamus. We found that NPW-like immunoreactive cell bodies were intensely found in the hypothalamus especially abundant in the arcuate and paraventricular nuclei and lateral hypothalamic area. Moreover, NPW-containing nerve fibers were found in apposition to orexin- and MCH-containing neurons in the lateral hypothalamus (6). These and our findings strongly suggest that NPW-containing neurons interact with orexin- and/or MCH-containing neurons in the hypothalamus and NPW participates in the regulation of feeding behavior in harmony with other feeding-regulating peptides.

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P31

cDNA CLONING AND GENE STRUCTURE OF HYPOCRETIN AND HYPOCRETIN RECEPTOR IN MEDAKA *Oryzias latipes*

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Hypocretin/orexin (HCRT) is a neuropeptide that is involved in the regulation of feeding and sleep in mammals (1,2). Recently, we immunohistochemically localized HCRT-like immunoreactivity in the brain and pituitary gland of medaka (3). However, little is known on the HCRT system in nonmammalian vertebrates including fish.

To obtain molecular basis on the roles of HCRT system in teleost, in the present study, we cloned cDNA encoding HCRT and HCRT receptor (HCRTR) in medaka and their gene structures were clarified.

The medaka HCRT cDNA (640 bp) is encoding 139 amino acid residues including HCRT-1 (orexin A) and HCRT-2 (orexin B). The medaka HCRT gene is composed of two exons and one intron.

HCRTR is a member of G protein-coupled receptor family with seven transmembrane domains. Two subtypes (HCRTR1 and HCRTR2) of HCRTR were classified to date. However, we found only a single HCRTR gene (HCRTR2) in the medaka genome. The medaka HCRTR2 cDNA (1348 bp) is encoding 420 amino acid residues. The HCRTR2 gene is composed of seven exons and six introns.

The site of HCRT and HCRTR2 expression and the effect of starvation on the expression of HCRT and HCRTR2 genes in medaka are now under investigation.

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P32

IDENTIFICATION OF THE REPTILIAN UNCOUPLING PROTEINS IN THE LEOPARD GECKO, *Eublepharis macularius*

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The ground living vertebrates had been adapted to the annual changes of the temperature in many ways. The most distinctive adaptation among them is nonshivering thermogenesis observed in endotherms. In mammals, this thermogenesis arises from the brown adipose tissues in response to cold exposure, and in birds, it occurs in the skeletal muscles. The main factors of these phenomenon are the uncoupling proteins (UCP), which are proton transporters localized in the mitochondrial inner membrane (1). To elucidate the molecular evolution of the endothermic mechanism, we focused on the UCP of the reptiles, which are ectothermic amniotes.

In this study, we identified partial sequences of leopard gecko (lg) UCP2 and UCP3. The amino acid sequences deduced from lgUCP2 and UCP3 cDNA show high similarities with their corresponding peptides of mammals and birds. The purine nucleotide-binding sites, which are involved in the control of the uncoupling activity of UCP, and mitochondrial carrier signature motives are well conserved in these deduced sequences. In the phylogenetic tree drawn with these sequences and their corresponding sequences of mammals, birds, reptiles and fishes, the leopard gecko clustered with reptiles and birds. By the RT-PCR, lgUCP2 was detected in the whole brain, liver, kidney, abdominal adipose tissue, and axillary adipose tissue. Whereas lgUCP3 was expressed in the whole brain, abdominal muscle and tail muscle. These expressional patterns are similar with mammals. In addition, synteny analysis of the UCP genes was performed using the squamate genome (green anole, *Anolis carolinensis*), and revealed the possibility of the deletion of reptilian UCP1. In conjunction with the recent view that thermogenic proton transport of mammalian UCP1 seems to be a late evolutionary characteristic and that ancestral UCPs may carry other substrates (2), these results suggested that the reptilian UCPs are good candidates for pleiotropic mitochondrial activities.

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P33

HEMOLYMPH MAJOR ANIONIC PEPTIDE (HemaP) DERIVED FROM THE SILKWORM, *Bombyx mori*, CAN INFLUENCE ON THE LARVAL FEEDING BEHAVIOR

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Regularly occurring feeding behavior in insects such as phytophagous caterpillars provided us some important questions “why and how insects start meal?”, even though they live on their diet. In the silkworm, *Bombyx mori*, the patterned feeding behavior was also observed in approximately two-hour rhythmicity (1). When some fraction of the partially purified *Bombyx* larval hemolymph by reverse-phase HPLC was injected, disturbance in the behavioral pattern was observed. Purification and structural determination revealed a peptidyl factor consisting of 62 amino acid residues, which showed no homologous sequences in the database. Then, we designated this peptide, Hemolymph major anionic peptide, HemaP.

Injection of HemaP into *Bombyx* larvae increased the levels of feeding, approximate digestibility (AD; a generally used digestive index), and duration in exploring around an artificial diet block. These results indicate that the hemal concentration of HemaP is related to feeding behavior in *Bombyx* larvae.

In addition, quantification of HemaP in the larval hemolymph by LC-ESI-TOF/MS revealed that HemaP level was increased by starvation. Interestingly, refeeding diet after starvation returned to the similar level of HemaP to that in the larvae fed *ad libitum*. Those results strongly indicate that the concentration of HemaP in the hemolymph can influence on the motivation of feeding behavior in *Bombyx* larvae by.

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IDENTIFICATION AND CHARACTERIZATION OF THE VERTEBRATE CALCITONIN ORTHOLOGOUS GENE IN THE ASCIDIAN, *Ciona intestinalis*

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Calcitonin (CT) is a hormone peptide essential for bone metabolism in mammals. The CT family peptides, including CT, CGRP, Adrenomedulin, Amylin, and CRSP, are conserved in vertebrates. However, no CT family peptides have ever been identified in invertebrates. The ascidian, *Ciona intestinalis*, which belongs to protochordates as a basal chordate, is closely related to vertebrates. The CT family signaling system is regulated by complicated combination of five CT family peptide ligands, two receptors, and three receptor-activity-modifying proteins. In this study, we identified *Ciona*-CT (Ci-CT) and, its putative receptor, Ci-CTR as the original components of CT family signaling pathway on the *Ciona* genomic database. *Ciona* has no paralogous genes, indicating that these genes are prototype genes of vertebrate counterparts.

RT-PCR analysis revealed that Ci-CT was expressed in the neural complex, endostyle, branchial sac, heart, and ovary, and that Ci-CTR was distributed in the neural complex, endostyle, intestine, and branchial sac. *In situ* hybridization analysis showed that Ci-CT was expressed in the whole of neural gland, whereas Ci-CTR was detected in the epithelium region of neural gland. Maircoarray analysis of Ci-CT-regulated genes in the neural complex revealed that several hormones, neuropeptides, their receptors, and neurotransmitter receptors were induced by Ci-CT. In contrast, Ci-CT was also shown to down-regulate the osmoregulatory gene and exocrine-associated gene, which are anticipated to be expressed in the neural gland. Functional analysis of these Ci-CT-regulated genes, will lead to elucidation of the functional evolution of the CT family.

Keywords: calcitonin, ascidian, *Ciona*, evolution

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ROLE OF NEUROHYPOPHYSEAL PEPTIDES IN CONTROLLING THE DRINKING BEHAVIOR OF THE EEL ACCLIMATED TO SEA WATER

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Role of teleost neurohypophysial peptides, vasotocin (VT) and isotocin (IT), in controlling the drinking behavior was examined. When VT was administrated into the posterior cardinal vein of the seawater eel, the drinking rate was suppressed significant- ly, while IT enhanced the drinking rate. Since the drinking rate of the eel is limited by contraction or relaxation of the upper esophageal sphincter (UES) muscle, effects of VT and IT were examined in the isolated UES muscle preparations. VT constricted and IT relaxed the UES muscle in a concentration-dependent manner, and the former effect was blocked by V1-receptor antagonist (H-5350) and the latter by oxytocin receptor antagonist (H-9405), suggesting presence of VT and IT receptors in this UES preparation. Similar relaxation were observed after treatments with IBMX, forskolin and 8BrcAMP and effect of 8BrcGMP was less than 1/4 of that of 8BrcAMP, suggesting that IT acts *via* cAMP production and that the relaxation *via* NO is unlikely. Even in scraped UES preparations, IT relaxed the UES. To clarify whether muscle cells or nerve terminals are target for IT, nerve activity was completely blocked by TTX. Even after blocking nerve activity with TTX, IT relaxed the UES muscle concentration-dependently, indicating direct action of IT on the muscle cells. On the other hand, IT enhanced the UES contraction evoked by nerve stimulation. This effect was also blocked by H-9405, suggesting that such enhancement is also mediated by IT receptor. Similar enhancements were observed after application of IBMX, forskolin and 8BrcAMP. However, carbachol- induced contraction was not enhanced by IT. In addition, when nerve activity and nerve-muscular transmission were blocked by TTX and curare respectively, the UES can be constricted by a stronger electrical stimulation (DC current) but the DC-current induced contraction was not enhanced by IT. These results suggest that IT acts nerve terminals and enhances release of acetylcholine. All together, it is plausible that IT may open the UES when the vagal nerve, which comes from the glossopharyngeal-vagal motor complex (GVC) in the medulla oblongata, is inhibited, while IT may close the UES when the GVC neurons are activated.

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SALINITY TOLERANCE AND EXPRESSIONS OF NEUROHYPOPHYSIAL HORMONE GENES IN FOUR SPECIES OF PUFFERS

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Two neurohypophysial hormones, vasotocin (VT) and isotocin (IT), are involved in osmoregulation and reproduction in teleosts. Our understanding of osmoregulatory mechanisms of VT and IT in fish is still fragmentary and needs a good animal model for elucidation of the mechanism. We chose puffer fish as an experimental model, because genomic resources of *Takifugu rubripes* and *Tetraodon nigroviridis* can be utilized to elucidate molecular mechanisms of osmoregulation.

To examine the adaptability of four species of pufferfish (*Takifugu rubripes*, *Takifugu niphobles*, *Tetraodon nigroviridis*, *Tetraodon fangi*) to different salinity environments, fishes were transferred to fresh water (FW), 10, 33 and 100% seawater (SW) and were kept for 3 days. Changes in blood osmolality, concentrations of Na^+ and Cl^-, Na^+/K^+-ATPase activity in the gill and kidney and expressions of VT and IT genes in the brain were examined during adaptation to different salinities.

In *Takifugu rubripes* transferred to the hypoosmotic conditions, blood osmolality, concentrations of Na^+ and Cl^- were decreased on 1 day after transfer (Day 1), but were maintained in the physiological levels during Day 2-3. The Na^+/K^+-ATPase activity in the kidney tended to increase in FW and 10% SW. The levels in expression of VT gene in FW tended to be higher than 100% SW on Day 1, while no significant changes were observed in the levels of IT mRNA. In *Takifugu niphobles*, the levels of VT and IT mRNAs in 33% SW were significantly lower than 100% SW at 12 hours after transfer. In *Tetraodon nigroviridis*, the levels of IT but not VT mRNAs were higher in 100% SW than FW and 33% SW. In *Tetraodon fangi*, all fish transferred to 100% SW died at 3 hours after transfer. The present results show that the expressions of VT and IT genes as well as salinity tolerance are different among the four puffer species, indication that puffer fish provides a useful experimental model for studying the osmoregulatory mechanisms of VT and IT.

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IDENTIFICATION AND PHYSIOLOGICAL ROLE OF VASOTOCIN PRECURSOR AND ITS RECEPTORS IN AFRICAN LUNGFISH, *Protopterus annectens*

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African lungfish, *Protopterus annectens*, living in freshwater, estivates to avoid dehydration of their body fluids in a subterranean mud cocoon during severe dry season. To clarify the mechanism of water and electrolyte balance of the lungfish maintained under the freshwater and estivation conditions, we focused on mechanisms of arginine vasotocin (AVT) known as an antidiuretic hormone in terrestrial tetrapods. We identified AVT precursor and its receptors (VT1R and VT2R) from the brain and kidney, respectively, and examined the mRNA expression of AVT precursor and the receptors in the hypothalamus and kidney of the lungfish maintained in the freshwater and estivation conditions.

Lungfish AVT precursor identified from whole brain was structurally similar to those of tetrapods and was higher identity with amphibian AVT precursor (68%) than that of teleost (57%). Phylogenetic analysis also showed that VT1R and VT2R of the lungfish were closely related to those of amphibian. The VT1R mRNA was abundantly expressed in the extrarenal tissues such as gill, lung and heart, moderately or low expressed in the brain, intestine and kidney. On the other hand, the VT2R mRNA was abundantly expressed in the kidney and heart, weakly expressed in the brain and ventral skin. Expression level of VT2R mRNA was a 100-fold than that of VT1R mRNA in the kidney. When lungfish were kept under dry condition for above 3 months, estivated lungfish significantly lost body weight, suggesting that their body fluid were decreased. Plasma osmolality, Na^+ and urea concentrations were significantly elevated relative to those of the lungfish maintained in freshwater. Furthermore, expression level of AVT mRNA was markedly increased in the hypothalamus and the changes in the expression level were correlated with significant increases of plasma osmolality in the lungfish kept in dry condition. Expression levels of VT1R and VT2R mRNA tended to decrease, but not significant, relative to those of the lungfish maintained in freshwater.

In the present study, we cloned for the first time the lungfish V2-type AVT receptor although the presence of the receptor has been questioned in fish. These results revealed that the AVT precursor and its receptors of the lungfish are structurally similar to those of tetrapods than teleost. Furthermore, the present study showed that the hypothalamic AVT mRNA expression might be stimulated in response to increase of plasma osmolality and/or cellular dehydration during estivation. Thus, osmoregulatory function of AVT in lungfish is probably related to the terrestrial adaptation of the ancestral tetrapods.

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ELECTROPHYSIOLOGICAL ANALYSIS OF TWO TYPES OF CHANNELS ACTIVATED BY cGMP IN EPITHELIAL CELLS FROM THE URINARY BLADDER OF JAPANESE TREE FROG, *Hyla japonica*

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Atrial natriuretic peptide (ANP) is probably involved in osmoregulation of amphibian. In our previous study, it is suggested that frog ANP (fANP) and cGMP stimulate amiloride-blockable Na^+ absorption through PKA-dependent pathway in the urinary bladder of the Japanese tree frog, *Hyla japonica,* using Ussing-type voltage clamp and whole-cell patch clamp experiments (1).

In the present study, transepithelial ion transport activated by both fANP and cGMP was investigated electrophysiologically using a patch-clamp technique in freshly obtained epithelial cells from the urinary bladder of *H. japonica*. Na^+ transport in the cell was confirmed to activate by both fANP and cGMP. Na^+ channel exhibited a low conductance for inward currents of 4.8 ± 0.2 pS, long open and closed times (c.a. 200 msec), and a positive reversal potential. The channel activity was decreased under presence of 10^{-6} M amiloride in the pipette solution. These characteristics were similar to those of amiloride-sensitive epithelial Na^+ channel (ENaC). Addition of 10^{-9} M fANP significantly increased the ENaC channel activity (NPo) from 0.72 ± 0.22 to 1.88 ± 0.46. On the other hand, mean amplitude and conductance of single channel did not change significantly after the addition of fANP. Addition of 10^{-5} M 8-Br-cGMP also significantly increased NPo from 0.56 ± 0.10 to 2.00 ± 0.33. The addition of fANP or 8-Br-cGMP failed to activate the ENaC under presence of 10^{-6} M amiloride. 8-Br-cGMP also failed to activate the ENaC under presence of a specific PKA inhibitor, KT-5720. On the other hand, 8-Br-cGMP activated K^+ channel that exhibits a high conductance (more than 20 pS). Addition of 10^{-5} M 8-Br-cGMP significantly increased NPo from 0.0007 ± 0.0004 to 0.0228 ± 0.0088. The channel is probably the cyclic nucleotide-gated (CNG) channel, which is non-selective cation channel. These results suggested that fANP and cGMP might activate Na^+ and K^+ transport via ENaC and CNG channel, respectively, in the epithelial cells of frog urinary bladder.

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EFFECTS OF ANGIOTENSIN II AND ARGININE VASOTOSIN ON WATER ABSORPTION RESPONSE IN JAPANESE TREE FROG, *Hyla japonica*.

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Anuran amphibians do not drink orally but absorb water osmotically across the high permeable ventral skin. During water absorption frogs display a characterized behavior termed the water absorption response (WAR). Previous studies demonstrated that angiotensin II (Ang II) facilitates water absorption in anuran species (1, 2). In dehydrated toads, plasma Ang II and arginine vasotocin (AVT) concentrations significantly increased along with rise of the plasma osmolality (3). However, it is less well clarified what mechanisms are involved in the WAR in anurans. The aim of the present study was to investigate effects of Ang II and AVT on the WAR in *H. japonica*.

Intracerebroventricular (ICV) injection of Ang II (1, 3, 10 pmol/g bw) significantly increased the duration of WAR in dose-dependent manner. Ang II type-1 receptor (AT_1R) antagonists, Candesartan and Losartan, inhibited the increasing of WAR caused by Ang II. These results suggest that Ang II promotes WAR through AT_1R mediated action. ICV injection of AVT (5, 10, 20 pmol/g bw) also increased the duration of WAR, and this effect was prevented by preinjection of mammalian vasopressin type-1 receptor (V_1R) antagonist OPC21268. Thus, AVT on the WAR probably functions via V1-type AVT receptor (VT_1R). In the next experiment, we examined hormonal interrelationships between AVT and Ang II on the WAR in *H. japonica*. Preceding injection of Candesartan to AVT inhibited the WAR caused by AVT. In adverse condition, OPC21268 did not inhibit the effect of Ang II. In the present study, both Ang II and AVT are involved in regulation of the WAR in *H. japonica*. It is suggested that the WAR induced by AVT may go through angiotensinergic pathways.

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P40

DIVERSITY OF ARGININE VASOTOCIN-DEPENDENT AQUAPORIN EXPRESSED IN THE VENTRAL PELVIC SKINS OF THE ANURAN AMPHIBIANS

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We have cloned three kinds of arginine vasotocin (AVT)-dependent aquaporins (AQPs) from the osmoregulatory organs from the tree frog, *Hyla japonica*. AQP-h2 protein was expressed in the ventral pelvic skin and urinary bladder, but not in the kidney (1) , whereas AQP-h3 displayed a specific distribution that was restricted to the ventral pelvic skin (2). We also revealed that AQP-h2K was predominantly expressed in the apical membrane or cytoplasm of the collecting duct principal cells (3). In response to AVT-stimulation, AQP-h2, AQP-h3, and AQP-h2K proteins are translocated to the apical plasma membrane for absorbing water through the membrane.

The response to antidiuretic hormone tends to be greater in the anuran species normally occupying drier habitats than those from wet habitats. Anurans are divided into four groups according to their habitats, *i.e.*, aquatic, semiterrestrial, terrestrial and arboreal species. AQP-h2 or AQP-h2-like protein was detected in the urinary bladder of all the species examined. The AQP-h2 homologue was detected in the pelvic skin of the terrestrial toad, *Bufo japonica*, as in the tree-adapted frog, *H. japonica*, but not in the other species. On the other hand, expression of the AQP-h3-like cDNA was identified in the ventral skin of all the frogs examined, from aquatic species to terrestrial dwellers, by molecular cloning. Therefore, AQP-h2 seems to be a urinary bladder-type AQP, while AQP-h3 seems to be a ventral pelvic skin-type AQP. It is possible that as anurans evolved into drier terrestrial environments, the urinary bladder-type AQP might have occurred in the pelvic skin, and resulted in absorbing water together with the ventral pelvic skin-type AQP.

Recently, our phylogenetic analyses of AQP proteins from anurans and mammals found that AQP-h2K belongs to the same clusters as mammalian AQP2, while AQP-h2 and AQP-h3 belong to an anuran-specific cluster (3).

In the present study, we also found that mRNA for the pelvic skin-type AQP (AQP-x3) in *Xenopus* is expressed in the pelvic skins, but the protein level is not detectable. The amino acid sequence of AQP-x3 protein is longer than the other pelvic type AQPs: the protein has the additional 10 amino acids in the C-terminal. When cRNA of AQP-x3-C272stop mutant or AQP-x3-C272S mutant was injected into *Xenopus* oocytes, AQP-x3 protein was translated and the volume size of *Xenopus* oocytes increased because the oocytes absorbed water. This finding suggests a possibility that normal AQP-x3 protein is degradated owing to the extension of the C-terminal 10 amino acid residues.

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P41

CHARACTERIZATION OF AN AQUAPORIN, AQP-H3BL, EXPRESSED IN MELANOTROPHS IN THE BULLFROG PARS INTERMEDIA

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Previously we have cloned one cDNA encoding a new aquaporin (AQP) homologous to mammalian AQP3 from the ventral pelvic skin of the tree frog, termed AQP-h3BL, and generated anti-peptide antibody (ST-184) against C-terminal region of the predicted protein (1). Immunolabels using this antibody was observed in the basolateral plasma membrane of several epithelial cells in osmoregulatory organs. We also found that positive labels for AQP-h3BL were present in melanotrophs of the pars intermedia in the bullfrogs.

We hypothesized that expression of AQP-h3BL protein is regulated by dopamine, and performed the following experiments. In the present study, we investigated the effects of the agonist (CB154) and antagonist (pimozide or haloperidol) of dopamine on expression of AQP-h3BL protein in the melanotrophs. Further, we examined the effect of $HgCl_2$, an inhibitor of AQP function, to α-MSH synthesis. The pituitary glands of adult bullfrogs were fixed with periodate-lysine-paraformaldehyde fixative, dehydrated with ethanol series, and embedded in Paraplast. Thin (4 μm) sections were cut and mounted on gelatin-coated slides. Immunoflurorescence staining was performed essentially as described previously (2) using rabbit anti-*Hyla* AQP-h3BL serum.

Immunopositive labels for AQP-h3BL protein were obtained in the pars intermedia of bullfrogs kept in a white background, but not in those of the bullfrogs kept in a black background. The black-adapted bullfrogs after the agonist-injection displayed white body-color with Melanophore Index (MI) 1, and melanotrophs in the pars intermedia were labeled for AQP-3BL. In additon, after injected with antagonists, white-adapted bullfrogs showed black color (MI 5), but labels for AQP-3BL for AQP-3BL were still observed in the melantrophs. Consequently, the expression of AQP-h3BL proteins may not be regulated by dopamine. Next, when the pars intermedia taken from the white-adapted bullfrogs (MI 1) were incubated with a cell medium containing $HgCl_2$ (0.3 mg/ml), the expression of proopiomelanocortin mRNA was not significantly different from that in the control group, suggesting that the expression of AQP-h3BL gene is not involved in synthesis of α-MSH. Other factors regulating the synthesis of α-MSH such as serotonin, noradrenalin, and GABA may be involved in the expression of AQP in the melanotrophs.

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P42

POSSIBLE HORMONAL REGULATION ON DISSOLUTION OF CALCIUM CARBONATE CRYSTALS IN THE BULLFROG ENDOLYMPHATIC SAC

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The amphibian endolymphatic sac (ELS) not only enlarges to form processes around the brain but also extends caudally along the vertebral canal and protrudes between the vertebratae, where it is referred to as the paravertebral lime sac. The lumen of these sacs contains many tiny crystals consisting of calcium carbonate, which are formed by otoconin-22 (OC22) protein. Previously, we cloned and sequenced a cDNA encoding bullfrog OC22 and found that calcitonin (CT) regulates the expression of OC22 mRNA in the ELS, thereby stimulating the formation of calcium crystals in the lumen of the ELS (1). In the present study, we investigated whether expression of vacuolar H^+-ATPase (V-ATPase) is regulated by any hormones, because the dissolution of the crystals is considered to be derived from a decrease in pH, adjusted by V-ATPase localized in mitochondria-rich cells in the follicular epithelium of the ELS.

To examine this issue, we used bullfrogs kept for one week under a container with water containing 0.8% $CaCl_2$ for forming the many crystals in the sac. After the treatment, ultimobranchial gland (UB) or parathyroid gland (PTG) was removed from the bullfrogs, and injected with CT or parathyroid hormone (PTH) plus vitamin D (Vit. D) for 3 successive days. Twelve hours after the final injection, we collected the ELS and performed RT-PCR and Western blot analyses to measure OC22 (1) and V-ATPase E-subunit (2) levels.

OC22 mRNA levels decreased after removal of UB, while the levels recovered after the supplement of CT. We failed to measure the OC22 protein levels, because the protein in the crystals was eliminated. However, the size of ELS recovered to the same levels as the sham-operated controls, suggesting OC22 protein was also expressed higher than that in the UB-removed controls. On the other hand, the removal of PTG also induced a decrease in the mRNA and protein expression of OC22, but a slight increase after supplement with PTH plus Vit. D, suggesting that expression of OC22 may increase by synergetic interactions of CT, PTH, and Vit. D. On the other hand, V-ATPase E-subunit mRNA levels were changed as similar to OC22mRNA, but the protein levels did not show a significant change. This finding may be related with the turnover of V-ATPase E-subunit protein. There was a significant difference in V-ATPase mRNA levels between PTH plus Vit. D-injected and control bullfrogs, suggesting a possibility that subunits other than E-subunit play a role as a regulator for expression of the V-ATPase function.

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CHANGES IN PROLACTIN GENE EXPRESSION IN AN AMPHIDROMOUS MIGRATORY TELEOST, AYU *Plecoglossus altivelis*

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Prolactin (PRL) is well known as a hyper-osmoregulatory hormone which promotes adaptation to fresh water but inhibits that to seawater, in a wide range of fish species. Ayu *Plecoglossus altivelis* is an annual and amphidromous fish, spending early life in coastal area over winter and ascending river in spring as juvenile. This fish has been used in anatomical, genetic, immunological, and physiological studies, repeatedly. Osmoregulatory action of PRL has been certified using this species as a hypernatremia after transfer from fresh water to seawater (1). To know the role of PRL in this migratory fish, we examined changes in mRNA levels of PRL during their down- and up-stream migrations in comparison with those of growth hormone (GH), which is known to be derived from a common ancestral molecule with PRL and possesses an opposite role in osmoregulation, seawater adaptation in euryhaline fishes.

In October, fries of ayu caught in the shore of Nezugaseki River (Tsuruoka, Yamagata) showed lower whole body contents of PRL mRNA, which were measured by real-time PCR and standardized by total RNA contents as pico mol/g RNA, than those in the stream. In contrast, GH mRNA contents increased significantly after the entrance from the river to the coastal area. In the shore, PRL mRNA maintained at low levels from March to mid April. Before the start of up-stream migration, the juvenile fish still remained in the coastal area showed ten-times elevations of PRL mRNA levels in late April and May. After the migration back to fresh water, mRNA levels of PRL were higher than those in seawater. The fish caught in the shore in late May and June showed moderate levels of PRL mRNA. During the up-stream migration, there was no significant change in GH mRNA contents. Results in this study suggest the osmoregulatory roles of PRL in ayu, inhibiting adaptation to seawater but promoting that to fresh water. Furthermore, a transient elevation of PRL mRNA levels just before the entrance into the river implied that PRL gene expression was already stimulated in the coastal area to prepare for the adaptation from seawater to fresh water.

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P44

ELEVATION OF THE PALSMA LEVELS OF INSULIN-LIKE GROWTH FACTOR-I (IGF-I) TRIGGERS HOMING OF CHUM SALMON OF JAPANESE STOCKS

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The previous study in masu salmon (*Oncorhynchus masou*), which has been used by us as a model fish in the neuroendocrine research of the molecular mechanisms of spawning migration, showed that IGF-I is important for the initiation of development of gonads, including gametogenesis and steroidogenesis, in early spring or rather late winter. Since the information from masu salmon provided many useful ideas on the neuroendocrine mechanisms of growth and sexual maturation, and spawning migration is instinctive behavior related to reproduction, we expected that IGF-I is also important for the initiation of gonadal development in oceanic chum salmon (*O. keta*). Further, such information would help understand when and how transition from growth to sexual maturation, or puberty, occurs in chum salmon in late winter and early spring, the pre-migratory period.

Chum salmon were sampled in the Gulf of Alaska in February, 2006, and in the Bering Sea during summer and autumn, 2003. On the basis of histology of gonads, they were divided into immature and maturing adult fish. The testes of maturing males showed spermatogenesis, whereas ovaries of females deposition of vittelogenin. Plasma was collected to determine the plasma levels of gonadotropins, IGF-I and steroid hormones, pituitaries for determination of gonadotropin contents and gene expression, and livers for determination of gene expression for IGF-I. Proteinaceous hormones were determined by heterologous coho salmon RIA systems, steroid hormones by EIAs, and gene expression was assessed by the amounts of mRNAs determined by quantitative real-time PCR assays.

The most important finding in the present study was that, in the Gulf of Alaska in February, chum salmon was dividable into immature fish and maturing adults even within the same age groups. In addition, body sizes of maturing adults were larger than those of immature fish, indicating those fish whose growth rates are high mature at least one year earlier than slowly growing fish. Coincidently, the plasma levels of IGF-I in the maturing fish in the winter Gulf of Alaska and the early summer Bering Sea were significantly higher than those in the immature fish in the winter Gulf of Alaska and the autumn Bering Sea. However, changes in the expression of IGF-I gene in the liver did not coincide with those in the plasma levels of IGF-I, probably because the liver is not a sole source of plasma IGF-I. Elevation of plasma levels of sex steroid hormones seemed to partially reflect initiation of activation of the pituitary-gonadal axis. IGF-I can be involved in this initiation of activation, and further decision whether homing or not homing in winter fish.

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GENE EXPRESSION FOR GONADOTROPIN-RELEASING HORMONES IN DISCRETE BRAIN LOCI OF PRE-MIGRATORY AND HOMING CHUM SALMON

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Neurons producing gonadotropin-releasing hormone (GnRH) are typical neuroendocrine cells that integrate information and regulate the pituitary-gonadal axis (PG-axis) and reproductive behavior (1). In chum salmon, activity of the PG-axis in terms of the amounts of gonadotropin mRNAs elevated by summer for motivation of spawning migration from the Bering Sea (2). In the present study, we examined expression of GnRH genes in discrete brain loci in pre-migratory chum salmon in the Bering Sea.

Chum salmon were caught along the 180^{o}-longitude line in the Bering Sea in summer and autumn from 2001 through 2003. They were divided into immature fish and maturing adult I and II on the basis of histological aspects of gonads (2). Brains were frozen in liquid nitrogen, and cut into 25μm thick serial transverse sections from the olfactory bulb (OB) through the saccus vasculosus with a cryostat. Every tenth sections were Nissl-stained to confirm cutting region, and other sections were separated into nine loci. Total RNAs were extracted from the brain sections and reverse transcribed. The amounts of mRNA encoding a precursor of salmon GnRH (sGnRH-II), which was dominantly synthesized in the forebrain of chum salmon (1), were determined by quantitative real time PCR. Furthermore, the amounts of mRNA encoding chicken GnRH-II (cGnRH-II) were determined.

The fish in the Bering Sea were mixtures of immature fish and maturing adults from June to July, while almost all of the fish were immature fish in September (2), indicating that the maturing adults left for the natal river by the end of summer. The amounts of sGnRH-II mRNA in all forebrain loci of the maturing adults were nonetheless not significantly different from those of immature fish in all years examined. The amounts of cGnRH-II mRNA in the midbrain tegmentum were also similar between the immature and maturing adults.

The present results indicated that expression of GnRH gene was not different between the immature fish and maturing adults in the Bering Sea during summer. We consider that activation of the PG-axis and the onset of spawning migration occur through interactions of various hormones including GnRH, sex steroid hormones (3) and somatotropic signals such as insulin-like growth factor-I (4). Future investigation of winter chum salmon in the Gulf of Alaska should be necessary, because the amounts of sGnRH mRNA elevated from winter through early spring in aquacultured-masu salmon (3).

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P46

ADULT CHUM SALMON POSSESS FRESH WATER ADAPTABILITY IN THE SUMMER BERING SEA

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The timing of runs of maturing Pacific salmon into the natal river varies among species and migration distances in the river. Masu salmon home to their natal river from March to May, when prolactin gene expression is stimulated prior to upstream migration (1). Since salmonids are not phylogenetically so distant, high sea salmons are expected to commonly possess hyperosmoregulatory ability prior to initiation of spawning migration. We therefore carried out FW-challenge test using adult chum salmon (*Oncorhynchus keta*) during the 2007 summer cruise of R/V *Hokko*-maru in the Bering Sea (25 June to 9 August).

Fish collected by angling were placed in an outdoor tank (1,000 L) on board, supplied with a continuous flow of seawater (SW, 34‰) under natural photoperiod. Running SW was replaced with FW (6‰) in FW-challenged group after sampling at 0 h, and thereafter a continuous flow of water was stopped and 200 L of water was renewed every 6 hours. Blood and gill samples were taken at -6 (initial control), 0, 12 and 24 h after replacement of water to analyze changes in plasma levels of electrolytes, testosterone (T), estradiol-17β (E2) and cortisol, and gill Na^+, K^+-ATPase activity.

Five out of 7 fish in FW-challenged group survived until 24 h after water replacement. The plasma levels of electrolytes in the FW-challenged group decreased within 12 h after the replacement, compared to the initial control and the control SW groups. The plasma cortisol levels increased in the FW-challenged group and decreased in the control group during 12 to 24 h. The plasma T levels transiently increased during 0 to 12 h, followed by decrease to the initial level by 24 h after the treatment. There were, on the other hand, no significant changes or differences in the plasma levels of E2 and the gill Na^+, K^+-ATPase activity in the control and the FW-challenged groups.

In conclusion, our present results indicate that summer oceanic chum salmon can survive for at least 24 h, suggesting that adult chum salmon possess FW adaptability even in the summer Bering Sea, although it is not clear whether the changes in the circulating levels of T and cortisol are involved in FW adaptation of summer oceanic chum salmon.

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P47

MEASUREMENT OF MELATONIN AND MELATONIN-RELATED INDOLEAMINES IN CYANOBACTERIA

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Melatonin (N-acetyl-5-methoxytryptamine, MEL), an indoleamine that was originally found in the vertebrate pineal gland, is synthesized enzymatically from serotonin (5-hydroxytryptamine, 5HT) by the sequential action of arylalkylamine N-acetyltransferase (AANAT) and hydroxyindole-o-methyltransferase (HIOMT). It is now widely accepted that, in vertebrates, MEL acts as an endogenous mediator of photoperiodic information and as a molecular component of the circadian time-keeping system.

Many previous studies have shown that MEL is not restricted to vertebrates, and is also present in various arthropods and recently higher plants. However, all of these organisms are eukaryotes. Then, to examine the presence of MEL and the synthetic pathway of MEL in cyanobacteria of prokaryotes, we used two cyanobacteria: *Nostoc punctiforme* PCC73102 (N.73102) and *Synechococcus elongatus* PCC7942 (S.7942). In these cyanobacteria, MEL and MEL-related indoleamines were measured by the reverse-phase HPLC with fluorescence detection. MEL was detected in the culture medium under normal conditions in N. 73102, on the other hand, 5HT as well as MEL was detected in S.7942. The amount of MEL in the medium of N. 73102 was much higher than that of S.7942.

To study enzymatic activities of AANAT and HIOMT in N.73102 and S.7942, the respective precursor compounds (5HT or 5-methoxytryptamine (5MTP) for AANAT; N-acetylserotonin for HIOMT) were added into the culture medium. In N.73102 AANAT activity was detected, while HIOMT activity was undetectable. On the other hand, both AANAT and HIOMT activities were detected in S.7942. In one hour incubation after addition of 5MTP, we found that MEL production (NAT activity) increased at 850 fold compared with the control medium in N. 73102. In S.7942, MEL production (NAT activity) in the medium containing 5MTP was at 10 fold higher than that in the control medium.

These results indicate that cyanobacteria (N.73102 and S.7942) have an ability of MEL production. Further study needs to elucidate the detail synthetic pathway of MEL in cyanobacteria.

P48

ANALYSIS OF OSTEOBLAST SPECIFIC MESSENGER RNA EXPRESSION IN THE REGENERATING SCALE OF GOLDFISH, *Carassius auratus*

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In most teleosts, it is known that the scale is a better potential internal calcium reservoir than the bone during periods of increased calcium demand, such as sexual maturation and starvation. Furthermore, teleost scale contains osteoblasts and osteoclasts similar to those found in avian and mammalian bone. We previously reported that osteogenesis in the regenerating scale is very similar to that of mammalian membrane bone, judging from morphological observations of the scale in goldfish (*Carassius auratus*). However, basic data concerning mRNAs expression of the osteoblast-specific genes in the regenerating scale are few.

To investigate changes of osteoblast specific mRNA expression during the scale regeneration of goldfish, we cloned several major osteoblastic markers and then examined the expression patterns of these osteoblastic markers using a quantitative real-time PCR. As a result, we succeeded in cloning of the osteoblastic markers: runt-related transcription factor 2 (Runx 2), osterix (OSX), type 1 collagen, alkaline phosphatase (ALP) and osteocalcin cDNAs from the normal scale of goldfish. This is the first report of the full coding sequence of OSX, ALP and osteocalcin molecules in goldfish. Then, the expression of these mRNAs was analyzed in the regenerating goldfish scale. We found that these markers were expressed in the regenerating scale and that the expression of OSX, type 1 collagen, ALP and osteocalcin mRNAs increased remarkably during the scale regeneration although Runx 2 mRNA did not change. Taking these results into consideration, the obtained osteoblastic markers in the present study should be useful for the study of osteoblasts in the teleost scale, especially during the scale regeneration.

P49

ESTABLISHMENT OF EXPERIMENTAL MODEL FOR BONE DISEASE USING THE SCALE OF GOLDFISH, *Carassius auratus*

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Teleost scales are essentially composed of type I collagen and calcium phosphate organized into hydroxyapatite. Similar to that found in human bone, both osteoblasts and osteoclasts existed on the scale regulate calcium homeostasis in teleosts.

This research focuses on two prospective experimental models of bone disease, i.e., autotransplantation of protein-denatured scales fixed by methanol and autotransplantation of scales with reversal of their polarity (upside down) in goldfish (*Carassius auratus*). Autotransplantion of the normal scales was used as a control group. On the fifth, tenth and fifteenth day after autotransplantation, tartrate-resistant acid phosphatase (TRAP) staining was performed on these scales to examine the differentiation of osteoclasts. Data collected on the fifth day reveal that osteoclasts appeared on the autotransplanted scales of both experimental groups. On the tenth day, in both groups, new regenerated scales were found adjacent to dermal side of each scale pocket and became larger in their sizes on the fifteenth day. However, protein-denatured autotransplanted scales were continuously resorbed by osteoclasts until the fifteenth day, while the upside-down autotransplanted scales were not. In the control group, few osteoclasts were found and none of regenerated scales were formed. Furthermore, using a quantitative real-time PCR the expression of the osteoclast-specific genes, i.e., nuclear factor of activated T cell c1, TRAP and cathepsin K, was examined in the protein-denatured scales on the third day after autotransplantation. The expression amounts of three osteoclast-specific genes were significantly ($p<0.001$) higher than those of the pre-autotransplanted scales.

These results suggest that autotrasplantation of protein-denatured scales is a possible model for osteolytic diseases.

P50

EFFECT OF TRIBUTYLTIN ON THE CALCIUM METABOLISM IN TELEOSTS AND ATTEMPT TO BIODEGRADATE BY MARINE BACTERIA

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Tributyltin (TBT) has been extensively used in antifouling paints on ships and fishnets. This causes widespread contamination of the marine environment. In marine Molluscan, for example, masculinization (imposex) has been observed on various seashores and adapted as a biomonitoring tool of marine pollution by TBT. In teleosts, as well as in Molluscan, it has recently been reported that TBT induced sex reversal of genetically female flounder to phenotypic males. As the sex hormone affects bone metabolism and participates in osteoblastic differentiation, we examined the effect of TBT on calcium metabolism in teleosts. Furthermore, biodegradation of TBT by marine bacteria was studied.

To examine the direct effects of tributyltin acetate (TBTA) on osteoclasts and osteoblasts, teleost scale, which has both osteoclasts and osteoblasts and is similar to mammalian membrane bone, was used in the present study. The activities of tartrate-resistant acid phosphatase and alkaline-phosphatase, as respective indicators of activity in osteoclasts and osteoblasts, were used. In freshwater teleosts (goldfish) and marine teleosts (nibbler and wrasse), the osteoclastic activity in the scales did not change as a result of TBTA treatment (10^{-9} to 10^{-5} M). On the other hand, the osteoblastic activity decreased in goldfish, nibbler, and wrasse after 6 hrs of incubation. In goldfish, even 10^{-10} M of TBTA significantly inhibited osteoblastic activity. The inhibitory activity in goldfish was stronger than that in nibbler and wrasse. Therefore, the mechanism was examined in detail using goldfish. The mRNA expressions of the estrogen receptor and insulin-like growth factor-I, which participate in osteoblastic growth and differentiation, decreased in the TBTA-treated scales. The mRNA expression of metallothionein, a metal-binding protein that protects the organism from heavy metal, increased much less than those of cadmium and methyl-mercury. Furthermore, we showed that the plasma calcium and hypocalcemic hormone (calcitonin) level increased in goldfish kept in water containing TBTA (10^{-10} and 10^{-8} M). The current data demonstrate that, in teleosts, TBT inhibits osteoblastic activity without affecting osteoclastic activity and disrupts the calcium metabolism, including the calcemic hormone, in goldfish.

In addition, we indicate the possibility that the strains of SK-2 (*Pseudoalteromonas sp.*) and SK-3 (*Pseudoalteromonas sp.*) degraded TBT when these bacteria were cultured in a seawater medium containing TBT (10 mg/L), peptone (0.1 %), and yeast extract (0.05 %). Plans are underway to examine in detail the biodegradation process of TBT by these marine bacteria and determine their culture conditions.

P51

DIRECT ROLE FOR GROWTH HORMONE IN REGULATING INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN 4 (IGFBP4) EXPRESSION IN CHICKEN

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We report the cDNA clone for the growth hormone (GH) dependent chicken insulin-like growth factor binding protein 4 (IGFBP4). Expression levels of the IGFBP4 mRNA in the chicken tissues was analyzed by Northern blot analysis. In the chicken IGFBP4, relatively high level expressions were observed in the liver, heart, lung, and testis. The expression level of IGFBP4 mRNA was negligible in the liver of sex-linked dwarf chicken, in which the GHR gene is defective because of a point mutation resulting in the generation of aberrantly spliced GHR mRNA. This result indicates that IGFBP4 mRNA is induced by GH in chicken. In addition to this *in vivo* studies, we employed cultured hepatocyte from chicken to demonstrate a possible direct regulation of IGFBP4 by GH. Significant effects on IGFBP4 mRNA was detected in GH-cultured hepatocyte. These results indicate that the expression of IGFBP4 mRNA in the chicken liver is depend on a GH signaling through GHR.

P52

A NOVEL PITUITARY TRANSCRIPTION FACTOR, PRX2 WAS ACTIVATED BY GNRH AND PLAYS SYNERGISTICALLY WITH EGR-1 ON LUTEINIZING HORMONE BETA SUBUNIT GENE

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We are currently attempting the cloning of transcription factors for porcine follicle-stimulating hormone β subunit (pFSHβ) gene by the Yeast One-Hybrid System with a porcine anterior pituitary cDNA library using the Fd2 region (-852/-746 b of pFSHβ promoter) (1) as a bait sequence. And finally, we had cloned *paired*-related homeobox factor 2, Prx2. Our recent studies revealed that Prx2 modulates the glycoprotein hormone α subunit and luteinizing hormone β subunit (LHβ) genes in addition to the FSHβ gene, indicating that Prx2 regulates the synthesis of FSH and LH at the transcriptional level as a common transcription factor. The present study, by focusing on a synergistic role of Prx2 with Egr-1 (early growth response factor-1) in the LHβ gene expression, and examined whether Prx2 responses to GnRH signaling.

In the pLHβ promoter, Prx2 binds to three regions composing an AT-rich sequence. One of the Prx2 binding sites, which is present at -90/-79 b from the transcription start site, locates between two Egr-1 binding elements (-101/-92 and -49/-41 b; essential elements for extracellular GnRH-action and are highly conserved in mammalian LHβ promoters) (2). Co-transfection assay using expression vector of Prx2 and Egr-1 showed synergistic activation of reporter genes fused with the region containing -1284/+7 by 40-fold, in CHO cells. Moreover, this activation required binding site of Prx2 or Egr-1.

We further tested whether GnRH could stimulate the endogeneous gonadotropin subunit, Egr-1 and Prx2 gene expression in LβT2 cells. The expression of all genes tested significantly increased by GnRH.

In summary, Prx2 plays synergistic activation with Egr-1 and its expression was regulated by GnRH. It is expected that Prx2 participates in a novel mediator of GnRH signaling.

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P53

DNA-BINDING CHARACTERISTICS OF TRANSCRIPTION FACTORS EXPRESSING IN THE ANTERIOR PITUITARY GRAND

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We have reported that Prop-1, Lhx2, Prx2 are candidates for transcription factors of the FSHβ gene. These three factors are predicted to play essential roles in the expansion of the pituitary primordium and the differentiation and development of the hormone-producing cells.

They are also homeobox factors which have homeodmain (HD) for DNA binding. In vitro DNA binding studies have demonstrated that most HD proteins bind to similar short consensus sequences containing a TAAT motif.

In this study, we have employed Systematic Evolution of Ligands by EXponential enrichment (SELEX) using random N15 oligomers and Electophoretic Mobility Shift Assay (EMSA) to expand and characterize the DNA binding sequences of these three factors and another pituitary transcription factor, Hesx1.

We found that Prop-1, Prx2 and Hesx1 which are paired class HD factors bind to TAATT/AATTA but only strongly Prop-1 binds to DNA by forming cooperative dimer.

Lhx2 is a LIM class HD factor and requires at least 5-6 bp of AT repeated sequence for DNA binding.

These results indicate that these factors well recognize similar AT-rich sequences, but have different DNA-binding characteristics from each other.

Further in vivo studies are required to determine these transcription factors' binding sites on target genes.

P54

NEURONATIN LOCALIZES IN SECRETORY GRANULE OF GONADOTROPE AND IS REGULATED BY STEROID HORMONES

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Neuronatin is a member of proteolipid protein family and two splicing variants (α- and β-form) are expressed in various tissues including the pituitary gland. The function is yet unclear, but a recent study reported that neuronatin is involved in glucose-mediated insulin secretion (1). This study performed the immunohistochemistry and immunoelectron microscopy to clarify the cellular and subcellular localization of Neuronatin in the pituitary. In addition, the effect of steroid hormones on the expression of neuronatin gene was examined by real-time PCR.

Immunohistochemistry showed that neuronatin localizes in cytoplasm of gonadotrope cells. Immunoelectron microscopy demonstrated that positive immunoreactants were only observed in the secretory granule of gonadotrope. Real-time PCR using total RNAs from rat pituitaries showed that the expression of Neuronatin gene was decreased by castration and was further decreased by the additional treatment of estrogen. On the other hand, the testosterone treatment after castration recovered its expression to the level of intact rat.

As described above, this study demonstrated that neuronatin localizes in the secretory granule of gonadotrope cells. Two steroid hormones regulate this gene positively (testosterone) and negatively (estrogen), indicating the presence of regulatory axis between pituitary and gonad.

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P55

MOLECULAR EVOLUTION OF PREPRO-THYROTROPIN-RELEASING HORMONE

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Thyrotropin-releasing hormone (TRH) is derived from a precursor protein, prepro-TRH (ppTRH) that contains multiple copies of the TRH progenitor sequence (-Lys/Arg-Arg-Gln-His-Pro-Gly-Lys/Arg-Arg-) and proteolytic cleavage at dibasic residues yields multiple copies of TRH. It is interesting to note that numbers of TRH progenitor sequences vary among vertebrates studied to date: chicken, 4; rat and mouse, 5; human and common carp, 6; *Xenopus laevis* and zebrafish, 7; sockeye salmon and goldfish, 8.

In the present study, we isolated ppTRH cDNA and characterized their deduced amino acid sequences from three classes of vertebrate, Chondrichthyes (brownbanded bambooshark *Chiloscyllium punctatum*), Reptilia (leopard gecko *Eublepharis macularius*) and Aves (zebra finch *Taeniopygia guttata*). Each ppTRH cDNA encodes a different number of TRH progenitor sequences (brownbanded bambooshark, 5; leopard gecko, 7; zebrafinch, 4). Comparison of deduced amino acid sequences among known vertebrate ppTRHs suggests that the ancestral ppTRH might have had eight TRH progenitor sequences. Several of these might be lost during the evolution due to mutation and/or deletion in the TRH-progenitor coding regions of ppTRH.

P56

AGNATHAN PITUITARY GLAND AND ITS EVOLUTIONARY IMPLICATION

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Lampreys and hagfish are particularly important animals to investigate the origin and the evolution of pituitary hormones, because they are only two extant representatives of the oldest class of vertebrates, Agnatha, arose about 530 million years ago. In this poster presentation, current knowledge on the agnathan adenohypophysial hormones is outlined.

The pituitary gland of lampreys exhibits more advanced features than that of hagfish, and resembles to that of gnathostome vertebrates. To date, four adenohypophysial hormones have been identified or cloned from the pituitary gland of sea lampreys. They are adrenocorticotropin (ACTH), melanotropins (MSHs), growth hormone (GH) and gonadotropin (GTH) (1-2). The topographic distribution of these four hormones within the lamprey pituitary gland is also comparable to that of gnathostome vertebrates (3). On the other hand, the pituitary gland of the hagfish exhibits very primitive features and is not well developed. It remains an enigma whether the hagfish pituitary gland contains tropic hormones of any kind. However, our recent study has revealed that at least GTH and ACTH are present in the hagfish pituitary gland (4-6). Namely, we have cloned cDNAs of both α- and β-subunits of GTH from the pituitary gland of brown hagfish, *Paramyxine atami* (6). Morphological exploration demonstrated that both GTH α- and β-subunits were completely synthesized in the same cells of the hagfish adenohypophysis (6). ACTH-like cells are also demonstrable in the pituitary gland of the brown hagfish. They were stained with anti-lamprey ACTH as well as several lectins (5). In the hagfish, GTH-like and ACTH-like cells are packed together in the same cell cluster, and thus each cell cluster appears to serve as a separate functional unit. These conditions of the hagfish pituitary gland seem to represent a primitive state before the differentiation of the pars intermedia (5). Finally, GTH and ACTH appear to be the original adenohypophysial hormones that appeared first during the evolution of the earliest vertebrates.

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P57

EFFECTS OF PITUITARY ADENYLATE CYCLASE-ACTIVATING POLYPEPTIDE (PACAP) AND MELANIN-CONCENTRATING HORMONE (MCH) ON SOMATOLACTIN (SL) RELEASE FROM THE GOLDFISH PITUITARY *IN VITRO*

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In the teleost pituitary, pituitary adenylate cyclase-activating polypeptide (PACAP) plays a role in mediating prolactin, growth hormone and gonadotropin release (1-3), and melanin-concentrating hormone (MCH) influences secretion of α-melanocyte stimulating hormone (4). In the present study, we examined the immunohistochemical relationship between PACAP and MCH nerve fibers and somatolactin (SL)-producing cells in the goldfish pituitary. Nerve fibers with PACAP- or MCH-like immunoreactivity were identified throughout the neurohypophysis, with their nerve endings also identified in the regions of the adenohypophysis, especially the pars intermedia that SL-producing cells exist. We also examined the effects of PACAP and MCH on SL release in goldfish pituitary using a cell immunoblot assay. This method allowed for the semiquantitative measurement of SL release from the cells. Incubation of the cultured pituitary cells for 3 h with graded concentrations of PACAP at doses of 10^{-9}-10^{-7} M provoked a dose-dependent increase in the immunoblot area for SL-like immunoreactivity (SL-LI). Treatment of pituitary cells with 10^{-8} and 10^{-7} M PACAP induced a significant increase in the immunoblot area for SL-LI. On the other hand, incubation of the cultured pituitary cells for 3 h with graded concentrations of MCH at doses of 10^{-11}-10^{-7} M provoked a dose-dependent decrease in the immunoblot area for SL-LI. These results suggest that PACAP and MCH can potentially function as a hypophysiotropic factor mediating SL release in the goldfish pituitary.

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P58

MOLECULAR CLONING AND CHARACTERIZATION OF cDNA ENCODING PITUITARY ADENYLATE CYCLASE-ACTIVATING POLYPEPTIDE FROM THE BRAIN OF FAR EASTERN BROOK LAMPREY

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Pituitary adenylate cyclase-activating polypeptide (PACAP) and growth hormone-releasing hormone (GHRH) are encoded by separate genes in mammals: PACAP is encoded with PACAP-related peptide (PRP), whereas GHRH and cryptic peptide are present in the same transcript. In non-mammalian vertebrates and protochordates, PACAP and GHRH have been believed to be encoded by the same gene and hence processed from the same transcript (1). Recently, genes encoding authentic GHRH were isolated and characterized from the zebrafish, goldfish and African clawed frog (2), suggesting that PRP-PACAP gene structure is conserved from teleosts to mammals. However, PACAP gene structure in the agnathans has not yet been examined. In this study, we isolated the PACAP cDNA from the brain of Far Eastern brook lamprey (*Lampetra reissneri*) to examine its structural characterization using RT-PCR method. PACAP and another peptide were deduced from the isolated PACAP cDNA. The primary structure of PACAP shares high sequence identity among known primary structures of PACAP in vertebrates and protochordate. Another peptide shares much lower level of sequence identity with known PRPs and GHRHs. These results suggest that in Far Eastern brook lamprey, PACAP cDNA also encodes another peptide.

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P59

CHANGES OF CORTICOTROPIN-RELEASING FACTOR EXPRESSION IN THE BRAIN OF THE BULLFROG, *Rana catesbeiana* DURING METAMORPHOSIS

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In submammalian vertebrates, corticotropin-releasing factor (CRF) acts as a potent stimulator of thyrotropin release. During metamorphosis in amphibians, plasma thyroidal and adrenocortical hormone levels are known to elevate almost simultaneously. Since adrenal corticoids potentiate the action of thyroid hormone, the increase of both hormone levels has been considered to facilitate metamorphic changes. To examine whether CRF is involved in the synchronized elevation of these two hormones through the enhancement of the release of thyrotropin and corticotropin, changes of CRF mRNA expression level and CRF-like immunoreactivity in the brain of the bullfrog larvae during metamorphosis were studied. While CRF mRNA levels in the whole brain of bullfrog were already high at premetamorphic stage, the expression of CRF mRNA in the diencephalon was same levels during the premetamorphic and metamorphic stages and it decreased at the end of metamorphosis. CRF-like immunoreactive neuronal cell bodies were observed in the mecencephalic (thalamic) regions invariably throughout the larval development. CRF-like immunoreactive cells were also found in the hypothalamus. The number of CRF-like immunoreactive cells in hypothalamus, but not in mecencepphalic region, showed a significant increase as metamorphosis progresses, and it reached a peak at metamorphic climax. The immunoreactive nerve fibers were observed mainly in the median eminence region and it became abundant as metamorphosis proceeds. The number of CRF-like immunoreactive cells in the hypothalamus as well as the density of immunoreactive fibers in the median eminence decreased simultaneously after metamorphosis was completed. These results suggest the involvement of CRF which was synthesized in the hypothalamus in the progression of metamorphosis.

P60

OPPOSITE MYOACTIVITY OF NOVEL NEUROPEPTIDES (FRamides) ENCODED IN THE SAME PREPROHORMONE GENE IN *Hydra*

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In the course of systematic identification of peptide signaling molecules combined with EST database from *Hydra*, we have identified a novel neuropeptide family that consists of two members with FRamide at the C-terminus; FRamide-1 (IPTGTLIFRamide) and FRamide-2 (APGSLLFRamide). The precursor sequence deduced from cDNA contained a single copy each of FRamide-1 and FRamide-2 precursor sequences. Expression analysis by whole-mount in situ hybridization showed that the gene was expressed in a subpopulation of neurons that were distributed throughout the body from tentacles to basal disk. Double in situ hybridization analysis showed that the expressing cell population was further subdivided into one population consisting of neurons expressing both the FRamide and Hym-176 (neuropeptide) genes and the other consisting of neurons expressing only the FRamide gene. FRamide-1 evoked elongation of the body column of epithelial *Hydra* that is composed of epithelial cells and gland cells but lacking all the cells in the interstitial stem cell lineage, including neurons. In contrast, FRamide-2 evoked body column contraction. The structure-activity relationship analysis showed that mature structures of FRamide-1 (9mer) and FRamide-2 (8mer) are important for their myoactivities. These results suggest that both of the neuropeptides directly act on epithelial cells as neurotransmitters and regulate body movement in an axial direction.

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EVOLUTIONARY RELATIONSHIP OF LPXRFa PEPTIDES AND PQRFa PEPTIDES - INSIGHTS FROM NOVEL CARTILAGINOUS FISH RF-AMIDE PEPTIDES

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In the RFamide peptide family, LPXRFa (X = L or Q) peptides act as hypophysiotropic hormones (1, 2). On the other hand, PQRFa peptides are known to act as neuromodulators of the opioid system (3). Interestingly, LPXRFa peptides and PQRFa peptides share similar C-terminal structures: LPXRFa peptides have C-terminal Leu-Pro-Leu/Gln-Arg-Phe-NH_2 motif and PQRFa peptides have C-terminal Pro-Gln-Arg-Phe-NH_2 motif. Furthermore, their receptors showed high identity at the seven transmembrane domain (about 70%) (4, 5). These observations suggest that LPXRFa peptides and PQRFa peptides may have diverged from a common ancestral gene. LPXRFa peptides and PQRFa peptides are present in the brain of teleosts and tetrapods. Our recent study demonstrated that at least PQRFa peptides are present in the brains of agnathans, the most ancient vertebrates (6). On the other hand, there is no evidence about the presence of neither LPXRFa peptides nor PQRFa peptides in cartilaginous fish. Because they serve as a critical outgroup in comparisons of tetrapods and teleosts, this study aimed to identify genes encoding LPXRFa peptides and PQRFa peptides in the brain of cartilaginous fish. We also demonstrated genome database analyses and provided some insights into the evolutionary relationship of LPXRFa peptide group and PQRFa peptide group.

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P62

PHYSIOLOGICAL EFFECTS OF PROLACTIN-RELEASING PEPTIDE (PrRP) ON BLOOD PRESSURE AND IMMUNE REACTION

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Prolactin-releasing peptide (PrRP) is an endogenous ligand of an orphan G-protein coupled receptor, hGR3/GPR10 and its immunoreactive cells are found to exist in dorsomedial hypothalamus (DMH), ventrolateral reticular nucleus (VLRN) and nucleus tractus solitarius (NTS) in the brain, and in the peripheral tissues of adrenal gland. PrRP is already known to have some functions like stress response, food intake regulation and prolactin secretion. Here, we would like to focus on its implication in the cardiovascular system as well as cell-mediated immunity as followed.

1), PrRP has already been reported to increase blood pressure. However, the mechanism remains to be defined. Therefore, we set out to study this aspect of PrRP. PrRP icv injection increases c-Fos expression in paraventricular nucleus (PVN) and locus coeruleus (LC), and we found c-Fos expressing cells in LC were noradrenalin cells, a good candidate for increasing blood pressure. This result shows PrRP acts on blood pressure through noradrenalin cells. Furthermore, AVP (arginine vasopressin) antagonist blocked AVP-induced increase in blood pressure and heart rate, but had no effect on PrRP-induced one. This means PrRP is not related to AVP in increasing blood pressure and heart rate.

2), PrRP is known to activate HPA axis and glucocorticoid level increase thereby. This glucocorticoid is known to have anti-inflammatory effect in the body. Therefore, we examined the effect of PrRP on cellular immunity. We induced allergic contact dermatitis (ACD) by oxazorone administration. In consequence, PrRP knock out mice (PrRP-KO) showed more ear swelling than wild type (WT) mice. This indicates PrRP suppressed inflammation through controlling cellular immunity.

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STRUCTURES AND DISTRIBUTION OF PROENKEPHALIN mRNAS IN BANDED HOUNDSHARK *Triakis scyllium*

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Met-enkephalin (Met-ENK), Leu-ENK, and β-endorphin (β-END) are opioid peptides exhibiting analgesic effects. Met-ENK, Leu-ENK, and β-END are derived from the precursor proteins proenkephalin (proENK), prodynorphin (proDYN), and proopiomelanocortin (POMC), respectively. These opioid peptides are suggested to have a common ancestry. We cloned POMC cDNA from the banded houndshark *Triakis scyllium* as a first step to elucidate the biological significance of opioid peptides in fish (1). Here, we undertook the molecular cloning of proENK and proDYN cDNA and examined the distribution of opioid peptide mRNAs in hypothalamus-pituitary systems.

Tissues were taken from *Triakis* captured off Misaki and kept for several weeks at the Ocean Research Institute, University of Tokyo. ProENK cDNA was amplified from brain cDNA with the polymerase chain reaction (PCR). ProDYN cDNA was cloned from a pituitary cDNA library prepared using the pGCAP10 vector or amplified from brain cDNA via PCR. PCR products were subcloned into the pT7 Blue-T vector. Nucleotide sequences were determined using a 3100-Avant Genetic Analyzer.

Triakis proENK cDNA was composed of 1150 bp excluding the poly A tail. PreproENK was deduced to comprise 265 aa, in which seven Met-ENKs were contained. The location of these Met-ENKs in *Triakis* proENK was similar to those in the galeoid shark, lungfish, and *Amia*. *Triakis* proDYN cDNA was composed of 1146 bp excluding the poly A tail. PreproENK was duduced to be 257 aa long, in which two Leu-ENK, two Met-ENK, and one Leu-ENK-like sequence were contained. The location of these hormonal segments in proDYN was similar to those in lungfish, bichir, eel, zebrafish, and tilapia. Reverse transcription PCR showed the expression of proENK in the hypothalamus, whereas the expression of proDYN was observed in the hypothalamus and pituitary. In the *Triakis* brain, both proENK and proDYN may serve as precursors for opioid peptides in the central nervous system as in other vertebrates. In addition, proDYN seems to provide ENKs as pituitary hormones for peripheral roles. Thus, the *Triakis* pituitary possesses a dual opioid system consisting of proDYN and POMC.

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THE CIRCADIAN REGULATION OF *IN VITRO* MELATONIN RELEASE FROM THE FISH PINEAL ORGAN: A COMPARATIVE STUDY

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In many fishes, the photoreceptive pineal organ harbors the circadian clock that regulates melatonin release in the pineal organ itself. However, the pineal organ of some salmonids did not exhibit circadian rhythms in melatonin release when maintained under constant darkness (DD) *in vitro*, suggesting that the pineal organs of all salmonids lack the circadian regulation of melatonin production. To test this hypothesis, the pineal organ of nine salmonids (*Coregonus lavaretus*, *Thymallus thymallus*, *Hucho perryi*, *Salvelius leucomaenis*, *Salvelius fontinalis*, *Oncorhynchus mykiss*, *O. keta*, *O. nerka*, and *Salmo trutta*), closely related osmerids (*Plecoglossus altivelis* and *Hypomesus nipponensis*) and two model teleosts (medaka *Oryzias latipes* and torafugu *Takifugu rubripes*) were individually maintained in flow-through culture under several light conditions.

Under light-dark cycles, the pineal organ of all species showed a rhythmic melatonin release with high rates during the dark phase. Under DD, pineal organs of osmerids, medaka and torafugu exhibited circadian rhythms in melatonin release with high rates during the subjective-night but the salmonid pineal organs constantly released melatonin at high rates. Under constant light, melatonin release was suppressed in all species. Thus, melatonin release from the pineal organ of osmerids, medaka and torafugu is regulated by both light and circadian clock but the circadian regulation is lacking in salmonids.

These results indicate that ancestral salmonids lost the circadian regulation of melatonin production during the evolution after the divergence from the other fish species.

P65

CONVENIENT METHOD FOR PREPARATION OF BIOLOGICALLY ACTIVE RECOMBINANT CHH OF THE KURUMA PRAWN, *Marsupenaeus japonicus*, USING THE BACTERIAL EXPRESSION SYSTEM

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Crustacean hyperglycemic hormone (CHH) is a neuropeptide synthesized by and secreted from the X-organ/sinus gland complex in medulla terminalis of the eyestalk in crustaceans. CHH exerts several biological functions, involving glucose metabolism, reproduction, molting, lipid metabolism, and stress response (1). CHHs and structurally related peptides comprise a peptide family, CHH-family. CHH-family peptides have been found only in arthropods so far. Among six CHHs characterized in *Marsupenaeus japonicus* (*Penaeus japonicus*-sinus gland peptide [Pej-SGP] -I-III, V-VII) (2), an expression system for recombinant Pej-SGP-VII (rPej-SGP-VII) has not yet been established. Therefore, we groped for a convenient preparation method of biologically active rPej-SGP-VII.

Here, we constructed an expression plasmid containing Pej-SGP-VII cDNA, by which rPej-SGP-VII was expressed in *Escherichia coli* as a fusion protein with N-terminal His-tag and Nus-tag. Through affinity-purification by a Ni-column, removal of N-terminal tags, and amidation at C-terminus, rPej-SGP-VII was obtained. Analysis of arrangement of intramolecular disulfide bonds revealed that the structure of rPej-SGP-VII was identical with native Pej-SGP-VII. Comparison of the CD spectrum of rPej-SGP-VII with that of native Pej-SGP-VII showed that they were almost identical. In addition, an *in vivo* assay confirmed that rPej-SGP-VII had authentic hyperglycemic activity. These data indicate that rPej-SGP-VII was successfully synthesized. Furthermore, this expression system for rPej-SGP-VII may be applicable to preparation of the other recombinant CHH-family peptides.

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P66

INSULIN SECRETION ABILITY OF BARFIN FLOUNDER, *Verasper moseri*

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Like in mammals, feeding induces insulin secretion in fish, but unlike in mammals, amino acids in fish are stronger insulinotropins than glucose (1). This suggests that the insulin secretion system in β-cells is different between fish and mammals. Plasma insulin level after feeding is related to neither the mRNA level of the insulin gene nor to the concentration of total insulin in principal islets (PIs) (2). The present study assessed the quantitative ability of insulin secretion to confirm the functional importance of the secretion stage in the regulation of insulin secretion compared to the transcriptional and translational stages of insulin production in fish.

PIs from flounders kept under various conditions were weighed and the concentrations of insulin in the PIs were measured using a time-resolved fluoroimmunoassay (TR-FIA) for barfin flounder insulin. Plasma insulin levels at various times after feeding in flounders fed fish or artificial feed were also measured using the TR-FIA. The half-life of barfin flounder insulin in plasma was measured using alloxan-treated barfin flounders injected intramuscularly with barfin flounder insulin.

The weight of PI was 76.3±23.8 mg/kgBW. Insulin concentration in PIs varied from 0.94±0.13 to 1.04±0.13 μg/mg. These results indicated that the amount of insulin in PIs of barfin flounder was about 80 μg/kgBW. The maximum plasma insulin level in flounders was 110 ng/ml. Plasma volume was thought to be less than 20 ml/kgBW in flounder; suggesting that the maximum insulin amount in plasma was less than 2.2 μg/kgBW. Therefore, barfin flounder stores insulin in PIs at over thirty-times higher than the maximum plasma level. The half-life of insulin in plasma was 4.1±0.4 hrs, which is twenty-times longer than that of mammals. The results in this study suggested the overwhelming dominancy of the secretion stage in the regulation of plasma insulin levels compared with transcriptional and translational stages in the insulin production system of flounder.

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HISTOLOGICAL ANALYSIS OF THE DISTRIBUTION OF CLUSTERIN IN THE RAINBOW TROUT

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In the rainbow trout (*Oncorhynchus mykiss*), the ultimobranchial gland secretes calcitonin (CT), which is involved in calcium metabolism. We have been studying this endocrine organ as a model system to understand molecular mechanisms for activating a particular gene and producing a peptide hormone abundantly. In the present study, we have characterized mRNAs expressed in the trout ultimobranchial gland by suppression-subtractive hybridization. Sequence analysis of 110 cDNA fragments indicated the existence of three types of keratins, Is (S8), IIe (E3), and IIs (S2), and ictacalcin, a calcium-binding protein reported to be expressed in the epidermis in mammals, suggesting that the CT cell may have characteristics of the epithelial cell. Cytoskeletal protein 4.1G was also identified. Furthermore, we found a secretory glycoprotein, clusterin, which is implicated in various physiological processes in other animals. RT-PCR analysis showed an amplified band for clusterin cDNA in each of the tissues examined in the rainbow trout, such as the ultimobranchial gland, thyroid, brain, pituitary, and intestine. In situ hybridization histochemistry localized strong expression of clusterin gene in the ultimobranchial gland, with the highest level in the sub-basal cytoplasm of the parenchymal cells located at the outermost region. Immunofluorescence staining with an anti-clusterin antibody revealed positive labels not only in the ultimobranchial gland, but also in the thyroid gland, gills, and intestine. At the cellular level, intense immunoreativity was detected at parenchymal cells in the ultimobranchial gland. Although immunopositive labels were observed at the follicular epithelial cells of the thyroid, at goblet cells in the intestine, and at mucous cells in the gills, clusterin mRNA has not yet been detected in these cells by in situ hybridization. The results suggest that the ultimobranchial gland is a major site of clusterin production. More study is needed to clarify the production sites of clusterin and to elucidate the physiological role of clusterin in the ultimobranchial gland.

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PARAFIBROMIN TUMOR SUPPRESSOR HAS ONCOGENIC PROPERTY IN THE CELLS EXPRESSING SV40 LARGE T ANTIGEN

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Primary hyperparathyroidism usually results from a single parathyroid adenoma, but in a minority of cases is part of hereditary syndromes, namely multiple endocrine neoplasia types 1 and 2A, familial isolated hyperparathyroidism, and hyperparathyroidism-jaw tumor (HPT-JT) syndrome. HPT-JT syndrome is characterized by parathyroid tumors, fibro-osseous lesions of the mandible and maxilla, and renal cysts and tumors. The gene whose inactivation is directly associated with the pathogenesis of HPT-JT syndrome has been identified as the tumor suppressor gene *HRPT2* (1). Parafibromin is a 531-amino acid protein encoded by *HRPT2*, a putative tumor suppressor gene recently implicated in the autosomal dominant hyperparathyroidism-jaw tumor familial cancer syndrome and sporadic parathyroid carcinoma. To investigate effects of parafibromin's overexpression on cell proliferation, we performed assays in four different cell lines. The transient overexpression of parafibromin inhibited cell growth in HEK293 and NIH3T3 cells, but enhanced cell growth in the SV40 large T antigen expressing-cell lines such as 293FT and COS7 cells. In 293FT cells, parafibromin was found to interact with SV40 large T antigen and its overexpression promoted entry into the S phase, implying that the interaction enhanced progression through the cell cycle. The tumor suppressor protein parafibromin acts as a positive regulator of cell growth like an oncoprotein in the presence of SV40 large T antigen.

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VISUALIZING EXOCYTOSIS IN RAT ANTERIOR PITUITARY CELL CLUSTER USING TWO-PHOTON EXTRACELLULAR POLAR TRACER (TEP) METHOD

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Exocytosis is the final step in peptide hormone secretion. Although this step is crucial in understanding the regulation of hormone secretion, it has been difficult to observe exocytosis in intact cells. TEP method (1,2) that combines post-fusion labeling for staining secretory granules and two-photon fluorescence microscopy for detection enables visualizing individual exocytotic events in intact cells by obviating cell manipulation including transfection. Single exocytotic event appears as a bright spot near the plasma membrane by this method. This spot represents the entry of extracellular fluorescence into granule through fusion pore of granule membrane and plasma membrane.

Pituitaries from male Wistar rats (8-10 week-old) were dispersed into cell clusters by collagenase treatment and mild pipetting. Normal human pituitary tissue was obtained with patient's consent during surgery for Rathke's cleft cyst. This normal tissue had to be removed to access the lesion. Human prolactin-secreting pituitary adenomas were obtained with patient' consent who underwent surgical treatment. These cell clusters were immersed in 0.5mM sulphorohdamine B for TEP. Rat pituitary cells had no spontaneous exocytosis at 6 hours after dissociation. GHRH (10^{-7}M) induced exocytosis in some cells. The secretory granules full-fused rapidly (few seconds). Most exocytosis occurred abvascularly rather than advascularly. These findings were similar to those in human normal pituitary clusters. LHRH (10^{-7}M) induced exocytosis in some cells in rat cell clusters. Full-fusion and kiss-and-stay mode were observed. Some human prolactin-secreting adenomas showed spontaneous exocytosis that was mostly kiss-and-stay type. It appears that each anterior pituitary cell type has peculiar pattern in terms of the mode of exocytosis.

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Key words: exocytosis, pituitary cells, rat, human

P70

OBSERVATION OF THE GAP JUNCTION AND LHRH PROJECTION ON FOLLICULO-STELLATE CELLS IN THE PARS TUBERALIS OF A RAT PITUITARY GLAND BY CONFOCAL LASER MICROSCOPY

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Luteinizing hormone-releasing hormone (LHRH) stimulates gonadotrophs via the hypophyseal portal vein system. Recently, Soji *et al*. (2004) have been proposing another pathway in which a network system of folliculo-stellate cells (FS cells) that are connected by the gap junction (GJ) modulates and/or supports the portal vein system (1). However, no morphological evidence that the FS cells are connected by the GJ from the pars tuberalis through the pars distalis has been shown.

Recently, Itakura *et al*. (2007) succeeded in generating transgenic rats (S100b-GFP rats) expressing green fluorescent protein under the promoter of the S100b protein gene that is a marker of FS cells (2). Utilizing these transgenic rats, the present study was designed to show the distribution of the GJ on FS cells by immunohistochemistry for connexin 43 (Cx43) and confocal laser microscopy. Furthermore, to determine whether the GJ is reconstructed between FS cells *in vitro*, we immunocytochemically observed Cx 43 in a primary culture of S100b-GFP rat's anterior pituitary cells.

Our observation indicated that FS cells interconnected the pars tuberalis and the pars distalis and that the GJ existed throughout these FS cells. We observed that the LHRH nerve projected not only onto the portal vein but also onto FS cells in the pars tuberalis. Furthermore, we identified that the GJ was constructed between FS cells *in vitro*. Our results supported Soji *et al.'s* hypothesis that FS cells have a role in transmitting LHRH signal from the hypothalamus to the gonadotrophs in the pars distalis.

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EFFECT OF EXOGENOUS E-CADHERIN OVEREXPRESSION ON PRIMARY CULTURE CELLS OF THE RAT ANTERIOR PITUITARY GLAND

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Cadherins are a family of cell surface glycoproteins that mediate calcium-dependent cell-to-cell adhesion, including epithelial (E-) cadherin, neuronal (N-) cadherin, and placental cadherin. We reported that E- and N-cadherin were coexpressed in the Rathke's pouch through the early development of the rat (1). However, in the adult rat, hormone-producing cells express only N-cadherin, while folliculo-stellate (FS) cells express only E-cadherin (2). We hypothesized that the change of cadherin types relates to the differentiation of hormone-producing cells and/or effects on the topography of hormone-producing cells and FS cells. In the present study, we observed the effect of exogenous overexpression of E-cadherin on hormone-producing cells and the topography of anterior pituitary cells in primary culture.

We used transgenic rats that express green fluorescence protein under the S-100b protein gene promoter, a marker of FS cells, donated by Professor Inoue of Saitama University. We cloned the full-length gene of rat E-cadherin and constructed E-cadherin expression-plasmids (p-rECDH). Primary culture anterior pituitary cells of the transgenic rats were transfected with p-rECDH by Lipofectamine 2000 (Invitrogen), dispersed by EDTA, and re-cultured. Expression of E-cadherin was determined immunocytochemically.

Folliculo-stellate cells and hormone-producing cells aggregated in clusters. Hormone-producing cells expressing E-cadherin were affected in their shape, but not in cell attachment. We may conclude that the expression of E-cadherin does not affect topographic cell affinities of hormone-producing cells.

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P72

MOLECULAR STRUCTURE AND EVOLUTION OF GONADOTROPIN RECEPTORS FROM MUMMICHOG *Fundulus heteroclitus*

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Gonadotropin receptors (GTHRs) belong to the glycoprotein hormone receptor, a family of G-protein coupled receptors. GTHRs have important roles in regulation of gonadal function by mediating gonadotropin signals to gonadal cells. In the present study, we determined the cDNA sequences and the deduced primary structures of the follicle stimulating hormone receptor (FSHR) and the luteinizing hormone receptor (LHR) from the mummichog (*Fundulus heteroclitus*), a useful model fish for reproductive biology, and compared them with those of various fish GTHRs. Both the mummichog FSHR (fhFSHR) and the LHR (fhLHR) had typical structural characteristics of the glycoprotein hormone receptor, such as the large extracellular domain (ECD), the transmembrane domain (TMD), and the intercellular domain (ICD). However, unlike most other vertebrate GTHRs, the fhFSHR had 10 leucine-rich repeats (LRRs). This unique structural characteristic was shared with perciform fish FSHRs. The phylogenetic tree based on the amino acid sequences of fish GTHRs showed that fish GTHRs are divided into 2 clusters, the FSHR cluster and the LHR cluster. The topology of the tree based on a whole amino acid sequence of the fish GTHRs was consistent with the well accepted taxonomical classification in the case of the FSHR cluster. However, it was not consistent in the case of the LHR cluster. Although the tree of the C-terminal half sequences (TMD and ICD) showed similar topology to that of whole sequences, the tree of the N-terminal half of fish GTHR sequences (ECD) showed different topology: the fhFSHR and perciform FSHRs were diverged from other fish FSHRs as soon as the fish FSHR was diverged from the fish LHR. These results suggest that the acanthopterygian ECD, which is responsible for the binding of the hormone, might have more rapidly evolved than other regions along with diversification of physiological properties of reproduction.

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MOLECULAR EVOLUTION OF THYROTROPIN-RELEASING HORMONE RECEPTORS

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Thyrotropin-releasing hormone receptor (TRHR) is a member of the G protein-coupled receptor family with seven transmembrane domains. Although the structure of the ligand, thyrotropin-releasing hormone, is conserved among vertebrates, the presence of three subtypes of TRHR (TRHR1, TRHR2 and TRHR3) is known to date. Thus, it is interesting to understand the molecular evolution of TRHR in vertebrates.

In this study, we isolated and characterized cDNA encoding TRHR in sockeye salmon (*Oncorhynchus nerka*) and medaka (*Oryzias latipes*). In addition, we predicted the structure of TRHR in the sockeye salmon by homology modeling.

Four different TRHR (TRHR1, TRHR2a, TRHR2b and TRHR3) were cloned from the sockeye salmon. The TRHR2b with only five transmembrane domains might be a splice variant of the TRHR2a. TRHR1a, TRHR1b, TRHR2 and TRHR3 were cloned from medaka. Analyses of deduced amino acid sequences and homology modeling predicted the three-dimensional structure of TRHR1, TRHR2a and TRHR3 of sockeye salmon.

Molecular phylogenetic analyses revealed that TRHR2 were first evolved form the ancient TRHR, and then TRHR1 and TRHR3 were differentiated. Furthermore, only mammals lacked the TRHR3 subtype, suggesting that mammals lost the TRHR3 during the evolution.

Further studies should be required to elucidate the difference in the ligand-receptor interaction among the three TRHR subtypes in vertebrates.

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THE FUNCTIONAL ANALYSIS OF THE TRANSCRIPTS DERIVED FROM HUMAN TYPE II GNRH RECEPTOR GENE

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Gonadotropin releasing hormone (GnRH) was originally identified as a hypothalamic decapeptide and stimulates pituitary gonadotropes to synthesize and release gonadotropins. Then it has the central role for controlling the reproductive function. Human possesses two GnRH ligands (GnRH-1, GnRH-2) and GnRH receptor genes (*GnRH-R1, GnRH-R2*). In the previous study, we used RNAi method to clarify the role of each receptor subtypes. It is noteworthy that at least two transcripts derived from *GnRH-R2* exists (GnRH-R2a, GnRH-R2b) and the established knocked-down cells (GnRH-R2a (-), GnRH-R2b (-)) showed altered effects of GnRH on cell proliferation. These results suggested that transcripts derived from *GnRH-R2* are functional although *GnRH-R2* was thought to be a pseudogene in human (1).

In this study, we examined the effect of two major transcripts derived from *GnRH-R2* to clarify GnRH receptor function, using the cell line which is enable to be knocked down the GnRH-R2a mRNA and GnRH-R2b mRNA simultaneously using RNAi method (GnRH-R2a/b (-)). As a model, we selected the TSU-Pr1, derived from human prostate cancer cell. And colony-forming efficiency assay was employed, which was established in our laboratory to easily evaluate the activity of GnRH on cell proliferation. In GnRH-R2a/b (-) cell, the effect of GnRH-2 was detected and Cetrorelix, which is known to be a GnRH-R1 antagonist, worked as an agonist. These results suggest that there are transcripts derived from *GnRH-R2* other than GnRH-R2a and GnRH-R2b. In future, it is required to examine the ligand selectivity on GnRH-R2a/b (-) cell and reconsider possible functional transcripts from *GnRH-R2*.

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P75

FUNCTIONS OF GnRH RECEPTOR HETERODIMERS OF THE ASCIDIAN, *Ciona intestinalis*

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GnRH is a ten-amino acid peptide hormone that plays pivotal roles in reproduction in vertebrates and octopus, and GnRH receptors are member of Class A (rhodopsin-like) G protein-coupled receptors (GPCRs). Recently, six GnRH forms (t-GnRH-3-8) and four GnRH receptor subtypes (Ci-GnRHR-1-4) were identified in the protochordate, *Ciona intestinalis*. t-GnRHs exhibit multiple cell-signaling activities including elevation of intracellular calcium ion and production of cAMP in HEK293 cells expressed individual Ci-GnRHR subtypes in ligand-receptor specific manners. Over the past decade, there is a growing body of evidence that GPCRs in native tissue *in vivo* form heterodimers acquiring ligand-binding affinity or selectivity, signal transduction, and internalization kinetics which are distinct from those of the corresponding monomers or homodimers. In the presentation, we show the functional modulation of Ci-GnRHR-1 via heterodimerization with the orphan receptor subtype, Ci-GnRHR-4. The dimerization between Ci-GnRHR-1 and R-4 in the membranes of the transfected HEK293 cells and *Ciona* tissues was detected by co-immunoprecipitation and immunoblot analysis. Binding assays using the membranes of the transfected HEK293 cells confirmed the binding of t-GnRHs to Ci-GnRHR-1 but not to R-4, and verified no alternation in ligand-binding affinity between Ci-GnRHR-1 homodimer and Ci-GnRHR1&4 heterodimer. Moreover, the heterodimer was found to elicit the elevation of intracellular calcium, extension of ERK phosphorylation, and up-regulation of cell proliferation in ligand specific manners, compared with the Ci-GnRHR-1 homodimer. In combination, these results indicated that Ci-GnRHR-4 is not an inactive receptor, but a modulatory component for Ci-GnRHR-1 in *C. intestinalis*.

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IDENTIFICATION OF cDNAS ENCODING THYROTROPIN-RELEASING HORMONE RECEPTOR FROM THE BULLFROG

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Thyrotropin-releasing hormone (TRH) has been proposed as a prolactin (PRL)-releasing factor in amphibians as well as in mammals. Indeed, TRH has been shown to stimulate the release of PRL from the bullfrog pituitary gland both *in vitro* and *in vivo*. Until now, however, the intracellular mechanisms that regulate PRL release and the receptor structures of TRH in amphibians are not known. The aims of the present study were to clarify the contribution of extracellular Ca^{2+} on TRH-induced PRL release and to characterize the cDNAs encoding the bullfrog TRH receptors (TRHRs).

The effect of TRH on the release of PRL was studied using a modified perifusion technique. Anterior lobes of the male bullfrog (*Rana catesbeiana*) pituitary glands were continuously perifused with a medium containing TRH as a secretagogue. The amount of PRL released was measured using a newly developed homologous enzyme immunoassay. TRH was found to stimulate the release of PRL from the perifused pituitary glands in a dose-dependent manner. In the presence of EGTA (2 mM) or Ni^{2+} (non-selective Ca^{2+}-channel blocker, 3 mM), the basal PRL release was decreased and the stimulatory effect of TRH (3×10^{-9} M) on PRL release was suppressed. In the presence of nifedipine (L-type Ca^{2+}-channel blocker, 10^{-5}M) also, the basal PRL release was decreased. These results suggest that both the basal PRL release and TRH-evoked PRL release from the bullfrog pituitary depend on the extracellular Ca^{2+}, and that L-type Ca^{2+}-channels may be involved in at least basal PRL release.

On the other hand, we isolated three distinct cDNA species encoding TRHR from the brain of the bullfrog. These three species were designated as fTRHR1, fTRHR2 and fTRHR3. Amino acid sequence and phylogenetic analyses revealed that the structure of fTRHR3 is closer to that of fTRHR1 than to that of fTRHR2. RT-PCR analysis revealed that the three bullfrog TRHR mRNAs have distinct patterns of expression. These observations suggest that each of the three TRHRs have specific functions that remain to be elucidated.

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INVOLVEMENT OF D2 DOPAMINE RECEPTOR SUBTYPE IN INHIBITION OF PROLACTIN RELEASE FROM THE BULLFROG PITUITARY GLAND

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Thyrotropin-releasing hormone (TRH) has been proposed as a prolactin (PRL) -releasing factor in amphibians as well as in mammals. Indeed, TRH has been shown to stimulate the release of PRL from the bullfrog pituitary gland both *in vitro* and *in vivo*. In mammals dopamine (DA) plays an inhibitory role in the control of PRL release from the pituitary gland. *In vitro* studies have revealed that DA likewise suppresses PRL release from the bullfrog pituitary gland. As receptor for DA, two types (D1 receptor and D2 receptor) have been reported. Until now, the intracellular mechanisms that regulate PRL release and the structure of DA receptors have not been elucidated in amphibians. The aims of the present study were therefore to clarify the subtypes of DA receptors that are involved in the DA-induced inhibition of PRL release and to characterize the gene structures of the bullfrog DA receptors.

The effect of the test substances on the release of PRL was studied by using a modified perifusion technique. The anterior lobes of the male bullfrog (*Rana catesbeiana*) pituitary glands were continuously perifused with a medium containing the test substances. The amount of PRL released was measured using a homologous enzyme-immunoassay. DA suppressed the TRH-inducible elevation of PRL release in a dose-dependent manner. Quinpirole (a D2 receptor agonist) also suppressed the stimulatory effect of TRH on the release of PRL. The inhibitory effect of DA on the TRH-inducible PRL release from the pituitary was nullified by the addition of L-741,626 (a D2 receptor antagonist) to the medium. In contrast, addition of SKF38393 (a D1 receptor agonist) was not associated with any suppressive effect on the TRH-induced PRL release, and that of SCH23390 (a D1 receptor antagonist) had no influence on the inhibitory effect of DA on the TRH-inducible stimulation of PRL release. These results suggest that the D2 receptor subtype mediates the inhibitory effect of DA on the TRH-induced PRL release from the pituitary glands.

On the other hand, two isoforms of the D2 receptor have already been identified in mammals. In this study, we isolated the mRNAs of three distinct D2 receptor isoforms from the brain of the bullfrog. RT-PCR analysis revealed that mRNAs of these three isoforms were apparently expressed in the bullfrog anterior pituitary. Further studies are required to elucidate the function of each isoform of D2 receptors in relation to the inhibitory effect of DA on PRL release.

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LOCALIZATION OF ARGININE VASOTOCIN V3/V1b TYPE RECEPTOR IN THE NEWT PITUITARY AND BRAIN

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In mammals, vasopressin V3/V1b receptor is predominantly found in the anterior pituitary and involved in adrenocorticotropic hormone (ACTH) release from corticotrophs (1). This receptor is also known to be expressed in the brain area, being involved in the control of social behavior.

Recently, we cloned newt (*Cynops pyrrhogaster*) arginine vasotocin (AVT) V3/V1b-type receptor cDNA for the first time in an amphibian. RT-PCR revealed that V3/V1b-type receptor mRNA was expressed mainly in the pituitary and brain, and this expression pattern was similar to that of mammalian vasopressin V3/V1b receptor (2).

As a step to elucidate the action of AVT via this type of receptor in the pituitary and brain, in situ hybridization using V3/V1b-type receptor specific probe was conducted. In the anterior lobe of the pituitary, V3/V1b-type receptor mRNA was specifically expressed in the ACTH cells. In the intermediate lobe, expression of this type of receptor gene was not observed. In the brain, V3/V1b type receptor mRNA was localized in several area of the diencephalon (ventral thalamus, dorsal hypothalamic nucleus, nucleus of the periventricular organ) and raphe nucleus of the medulla oblongata. Notably, in the raphe nucleus, mRNAs of V3/V1b type receptor and serotonin transporter that is known to be a marker of serotonin neuron were revealed to be colocalized in the raphe nucleus.

The results obtained in the newt indicate that V3/V1b-type receptor in the pituitary mediates the release of ACTH as in the case of mammals and raise the possibility that in the medulla oblongata it regulates serotonin neuron functions.

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P79

IDENTIFICATION OF GHRELIN RECEPTOR-LIKE RECEPTOR AND ITS GENE STRUCTURE IN RAINBOW TROUT

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Ghrelin is a GH-releasing and orexigenic peptide released from the stomach. The receptor is a G-protein-coupled, growth hormone secretagogue-receptor (GHS-R), and two types of receptor, a functional GHS-R1a and function-unknown splice variant GHS-R1b, have been known. In non-mammalian vertebrates, these receptors have been identified in chicken, pufferfish, seabream and zebrafish. In this study, we identified ghrelin receptor-like receptor (GHSR-LR) in rainbow trout. We isolated two mRNA: one encodes a 387-amino acid protein, which is similar to GHS-R1a (GHSR1a-LR), and the other encodes a 300-amino acid GHS-R1b-like protein (GHSR1b-LR). Trout GHS-R1a-LR showed 71, 67, 60, 58% identity with seabream, pufferfish, chicken and rat GHS-R, respectively. GHS-R1a-LR mRNA predominantly expressed in the pituitary, and followed by the brain, intestine, head kidney and heart. GHSR1b-LR mRNA also expressed predominantly in the pituitary, and followed by the brain, stomach, spleen, intestine and head kidney. The GHSR-LR gene was composed of two exons separated by an intron, as seen in other animal species, and GHS-R1b-LR was considered to be generated an alternative splicing of the gene. Functional analyses of the GHSR1a-LR were conducted using human embryonic kidney (HEK) 293 cells, but expected intracellular calcium increase could not observe. We found that full-length of receptor proteins might be not generated in the cells because unexpected mRNA splicing was occurred. In conclusion, further studies is necessary to conclude our identified GHS-R1a-LR as functional ghrelin receptor in rainbow trout, although the gene structure and characterisation of protein sequence are highly conserved among other GHS-R.

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IDENTIFICATION OF GHRELIN RECEPTOR-LIKE RECEPTOR AND ITS GENE STRUCTURE IN RAINBOW TROUT

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Ghrelin is a GH-releasing and orexigenic peptide released from the stomach. The receptor is a G-protein-coupled, growth hormone secretagogue-receptor (GHS-R), and two types of receptor, a functional GHS-R1a and function-unknown splice variant GHS-R1b, have been known. In non-mammalian vertebrates, these receptors have been identified in chicken, pufferfish, seabream and zebrafish. In this study, we identified ghrelin receptor-like receptor (GHSR-LR) in rainbow trout. We isolated two mRNA: one encodes a 387-amino acid protein, which is similar to GHS-R1a (GHSR1a-LR), and the other encodes a 300-amino acid GHS-R1b-like protein (GHSR1b-LR). Trout GHS-R1a-LR showed 71, 67, 60, 58% identity with seabream, pufferfish, chicken and rat GHS-R, respectively. GHS-R1a-LR mRNA predominantly expressed in the pituitary, and followed by the brain, intestine, head kidney and heart. GHSR1b-LR mRNA also expressed predominantly in the pituitary, and followed by the brain, stomach, spleen, intestine and head kidney. The GHSR-LR gene was composed of two exons separated by an intron, as seen in other animal species, and GHS-R1b-LR was considered to be generated an alternative splicing of the gene. Functional analyses of the GHSR1a-LR were conducted using human embryonic kidney (HEK) 293 cells, but expected intracellular calcium increase could not observe. We found that full-length of receptor proteins might be not generated in the cells because unexpected mRNA splicing was occurred. In conclusion, further studies is necessary to conclude our identified GHS-R1a-LR as functional ghrelin receptor in rainbow trout, although the gene structure and characterisation of protein sequence are highly conserved among other GHS-R.

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MOLECULAR CHARACTERIZATION OF CHICKEN MOTILIN RECEPTOR (MTL-R)

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G-protein coupled receptor 38 (GPR38 / MTL-R) belongs to the ghrelin receptor superfamily (1), and has been characterized as specific receptor of gastrointestinal peptide, motilin (2). However, molecular characterization of MTL-R has been performed only in mammals and fish. In this study, we performed molecular characterization for chicken MTL-R (cMTL-R) and profiled its mRNA expression in chicken tissues. Total RNA was extracted from chicken tissues and a fragment of cMTL-R cDNA was cloned after the amplification by RT-PCR using primers designed from the putative cMTL-R gene sequence appeared in the chicken genome database. The cDNAs for 3'- and 5'- regions were cloned by RACE PCR. The cloned full-length cMTL-R cDNA was about 1.7 kb in length and encoded 349 amino acids. The amino acid sequence showed a significant homology to mammalian MTL-Rs (>59%). Realtime PCR analysis revealed that cMTL-R mRNA was expressed in all tissues examined with high levels in digestive organs and oviduct. The expression levels of cMTL-R in the proventriculus and duodenum were rapidly decreased after hatching. Functional analysis revealed that chicken motilin increased intracellular Ca^{2+} concentration in HEK293 expressed cMTL-R, but chicken ghrelin did not. These results indicate that identified cMTL-R is specifically activated by motilin and might be involved in peristaltic motion in digestive and reproductive ducts in chicken.

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CHARACTERIZATION OF SIGNALING PATHWAYS THROUGH PROTON-SENSING G-PROTEIN-COUPLED RECEPTORS

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Extracellular acidification has been shown to induce a variety of responses at the whole animal, tissue, and cellular levels. In vascular systems, for example, acidosis causes vasodilation of systemic circulation at whole animal levels, relaxation in isolate vessels and alteration of a variety of cellular activities at the cell levels, including cAMP accumulation and changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). However, the mechanism by which extracellular acidification modulates the cellular activities has not fully been characterized. OGR1, GPR4, G2A, and TDAG8 share about 50 % homology with each other and form a family of G-protein-coupled receptors (GPCRs). Recent studies have revealed that the GPCRs sense extracellular protons and modulate some cellular activities. In this study, we examined the signaling pathways of GPR4, which is a member of proton-sensing GPCRs.

Cellular activities, including inositol phosphate production, cAMP accumulation, the epidermal growth factor (EGF)-induced extracellular signal-regulated kinase (ERK) acitiviy, the serum response element (SRE) promoter activity and nuclear factor of activated T cell (NFAT) promoter activity, in response to extracellular acidification were measured in HEK293 cells or RH7777 cells, which were transfected with human GPR4 plasmid. Specific inhibitors or dominant negative constructs for intracellular signaling pathways were employed to characterize the acidification-induced cellular activities.

Extracellular acidic pH reduced EGF-induced ERK activity in association with increased cAMP accumulation. Acidification stimulated the SRE activity in a manner dependent on G_{13} protein/Rho signaling pathway. Acidification also stimulated the NFAT activity in association with inositol phosphate production. Our experimental results suggest that GPR4 may be a multi-functional receptor coupling to G_s, G_{13}, and $G_{q/11}$ proteins in response to extracellular acidification.

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CLONING AND CHARACTERIZATION OF THE ECDYSONE RECEPTOR AND ULTRASPIRACLE PROTEIN FROM THE WATER FLEA, *Daphnia magna*

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By screening the *Daphnia magna* cDNA library (1) and combination of 5'-RACE, cDNAs encoding the ecdysone receptor (EcR) and ultraspiracle (USP) protein were obtained. We isolated three isoforms of EcR that differ in the A/B domain (EcR-A, B1, B2). Phylogenetic analysis indicated that *Daphnia* EcR is most similar to EcRs from other crustaceans, and that *Daphnia* USP is most similar to mammalian RXRs rather than to USPs from insects such as the *Diptera* or *Lepidoptera*. Quantitative PCR analysis demonstrated that the expression of EcR-A correlated well with the timing of molt.

We constructed a two-hybrid system as a reporter for ligand-dependent activation of EcR from *Daphnia* and *Drosophila*. The gene fusions encoded the EcR lignad-binding domain (LBD) fused to the Gal4 DNA binding domain, and the USP-LBD fused to the Vp16 activation domain. These chimeric genes were transfected into Chinese hamster ovary (CHO) cells with a luciferase reporter gene. Dose-dependent activation of the reporter gene could be observed for both *Daphnia* and *Drosophila* EcR reporters when transfectants were exposed to ecdysone analogs such as ponasterone A, muristerone A and tebufenozide. This two-hybrid system may represent a useful reporter system for further examination of hormonal and chemical effects on *Daphnia* at the molecular level. This study was supported by Grants-in-Aid for Scientific Research from Ministry of Education, Culture, Sports, Science and Technology, and grants from the Ministry of Environment, Japan, and a grant of LRI by JCIA.

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3S-1

PRELUDE TO REGENERATION ----MECHANISMS OF CELL DEATH----

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Regeneration can be classified based on morphological criteria into two categories : pathological(accidental or traumatic) and physiological(repeat or repair) regeneration. In either category, the first reaction is cell-death and cell-elimination in the wound area . There are two types of cell death: necrosis and programmed cell death. Necrosis is characterized by cell swelling and membrane bursting. Programmed cell death plays an important role for the formation and maintenance of tissue architecture in multicellular organisms. It regulates the sculpting of structures, deletion of vestigial organs, and maintenance of proper cell number. Programmed cell death may be classified on the basis of morphological changes into two types. The most extensively studied one, called apoptosis, is characterized by membrane smoothing, cell shrinkage, DNA fragmentation and preservation of organelles. The other one, autophagic cell death or type II programmed cell death, is characterized by the appearance of vacuoles engulfing bulk cytoplasm and organelles such as mitochondria and endoplasmic reticulum. Allografts in human or mouse, for instance, are chronically rejected by apoptosis with DNA fragmentation, while rejection of allografts in terrestrial molluscs, degeneration of insect muscle, degeneration of eye or copulatory apparatus in planarians are induced by autophagic cell death. In the final process of autophagic cell death, pycnotic nucleus left in hemocoel is cleared away by heterophagy. Cell –death genes exist from nematode to human with high homology. This fact indicates that the genes may be conserved across phylogenic lineages. Thus, programmed cell death in regeneration system is considered to occur in two different pathways: apoptosis and autophagy.

3S-2

THE ORIGIN OF STEM CELLS IN THE REGENERATING MARINE AND FRESHWATER PLANARIANS

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Planarians have high regenerative ability. It has been reported by many researchers that this ability resides in the neoblasts (pluripotent/totipotent stem cells). Neoblasts are considered to be the only proliferating cells in asexual organisms, and they can renew by themselves and undergo differentiation to any cell types. Electron microscopic studies have shown that the neoblast has many chromatoid bodies in the cytoplasm.

Recently our group found that the intestinal cells of *Pseudostylochus intermedius* (polyclad) include mitochondrial large ribosomal RNA (mtlrRNA) , mitochondrial small ribosomal RNA (mtsrRNA), chromatoid bodies and abundant free ribosomes, and have proliferative ability. Furthermore, we obtained the figures that the neoblast-like cells with signals for mtlrRNA and mtsrRNA might have derived from the regenerating intestine and dispersed in the new tissue. Using EM *in situ* hybridization technique, we previously revealed that the mtlrRNA and mtsrRNA were localized on the surface of the chromatoid bodies in the embryos of *P. intermedius* (1). MtlrRNA and mtsrRNA have been identified as the factor required for the formation of the germline progenitors in *Drosophila* (2).

Regarding to the origin of neoblast, there exist two theories, that is, the embryonic stock cell theory and the dedifferentiated cell theory. I support both theories and consider as follows. 1. In the species reserving neoblasts abundantly in the parenchyma, the dedifferentiated cells may not play the role of cell supply in epimorphosis. However, in the species not reserving so much, the dedifferentiated cells may also take part in their regeneration. 2. There is a possibility that the intestinal cells have a pluripotency and have the ability to regenerate themselves, and can supply the neoblasts in marine planarian polyclads.

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3S-3

CELLULAR AND MOLECULAR MECHANISMS OF NERVE REGENERATION OF HYDRA

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The developmental dynamics of the nerve cells are unique. Neurons are produced continuously by differentiation from interstitial multipotent stem cells. These neurons are continuously displaced outwards along with epithelial cells and are sloughed off at the extremities. However, the spatial distribution of each neural subset is maintained. Mechanisms related to these phenomena, of the position-dependent changes of neural phenotypes were proposed.

Hydra possesses several advantages for the study of nerve net formation. We can examine nerve net formation in various unique experimental systems: the regenerating, repopulating, budding, and normal system. Nerve net formation progresses under different conditions depending on the system. Therefore, we can clarify important factors in the cellular level by comparing nerve net formations.

In the course of large scale screening of peptide signal molecules, peptide molecules related to nerve differentiation have been identified. The LPW family, composed of four members sharing common N-terminal, L(or I)PW, inhibits nerve differentiation in hydra. In contrast, Hym355 (FPQSFLPRG-NH3) activates nerve differentiation in hydra. LPWs are epitheliopeptides, whereas Hym355 is a neuropeptide. Our preliminary results about study to clarify the exact site of the action suggest that it is commitment, but neither migration, nor differentiation.

3S-4

ADULT NEWT RETINAL REGENERATION: IS FGF AN INDUCTION FACTOR OF REGENERATION?

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The retina is a part of the central nervous system in the eye, and disease or traumatic injury of the retina has a serious impact on normal life. Recent biomedical studies have revealed that pigment epithelium (PE) cells of the adult eye, which transdifferentiate into retinal cells *in vitro*, are one of the most hopeful cell sources for future cell therapy of degenerated retinas. However, unfortunately, we do not have clinical strategies to regenerate the whole retina from PE cells in the eye of a patient. In contrast, some urodele amphibians such as newts can regenerate the entire retina through transdifferentiation of the retinal PE (RPE) cells even when the neural retina is completely lost. Therefore, urodelian retinal regeneration can be an *in vivo* transdifferentiation model of PE cells to gain critical information aimed at establishing new clinical strategies for retinal regeneration. Thus far, we have studied the cellular and molecular mechanisms of adult newt retinal regeneration, and currently focus on the induction mechanism of retinal regeneration. In embryonic and larval vertebrates, an FGF2/FGFR-2/MEK/Pax6 pathway has been suggested to be involved in the induction of RPE transdifferentiation into a neural retina. Here we show that 1) the adult newt RPE cells transdifferentiate into neural cells *in vitro* through activation of an MEK pathway, 2) FGF2 promotes de-differentiation and proliferation of RPE cells but is unlikely a primary induction factor, 3) *FGFR-2* and *Pax6* genes do not express in the adult newt RPE cells, 4) both genes are expressed upon removal of the neural retina, 5) neural transdifferentiation of RPE cells is inhibited in the initial process of retinal regeneration. These results suggest that the retinal regeneration of adult newt is induced via a mechanism different from embryonic/larval one and the RPE transdifferentiation is controlled elaborately *in vivo*.

3S-5

NEUROGENESIS AND REGENERATION IN THE MAMMALIAN BRAIN

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For more than 100 years, it was believed that neurons are never generated in the adult brain. This paradigm changed in the late 90's and most neuroscientists now believe that neurons continue to be generated in two restricted adult brain regions: the hippocampus and the subventricular zone of the forebrain. The phenomenon of adult neurogenesis itself had been discovered in the early 1960's by Altman. However, his studies were not appreciated and unregarded. In the early 90's we found that newly generated and developing neurons in the adult hippocampus specifically express polysialic acid (PSA), a carbohydrate portion of neural cell adhesion molecules (NCAM). The immunohistochemistry for PSA can visualize the detailed morphology of the dendrites and axons of developing neurons. In the late 90's the adult neurogenesis was demonstrated in the human hippocampus, and the concept of the adult neurogenesis is widely accepted.

Recently, progenitor cells of the adult neurogenic regions have been reported to possess astrocytic features and express GFAP. However, the questions remain how astrocytic progenitors divide and differentiate into neurons, and how the neuroblasts migrate to the GCL. To observe these developmental courses, we developed a time-lapse imaging system with mouse GFAP promoter-controlled enhanced green fluorescent protein (GFAPp-EGFP Tg mice, kindly provided by Dr. Shioda, Showa Univ.) or with an EGFP-retrovirus labeling. These results show that GFAP-positive neural progenitor cells differentiate into neurons via intermediate cells expressing both astrocytic and neuronal markers. Furthermore, neuronal proliferating progenitors make clusters, and most postmitotic neuroblasts migrate horizontally from the clusters, extending tangentially oriented processes, and finally extend apical dendrites. These results suggest that the neural progenitor cells have a systematic cellular arrangement and intercellular interaction during their development.

3S-6

REGENERATION AND RESORPTION OF THE GOLDFISH SCALE AND ITS REGENERATION BY MELATONIN

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Different from mammals, most teleosts have the scales which contain as much as 20% of the total body calcium and are a better potential internal calcium reservoir than the vertebral bone during periods of increased calcium demand, such as sexual maturation and starvation. Furthermore, the teleost scale contains osteoblasts and osteoclasts similar to those found in mammalian bone. In addition, we recently demonstrated that the osteogenesis of the regenerating scale is very similar to that of mammalian membrane bone. Judging from these morphological studies, we strongly suggest that the teleost scale should be a good model for the study of osteogenesis and calcium metabolism.

To clarify the function of osteoclasts and osteoblasts in the scale of goldfish (*Carassius auratus*) during the scale resorption and regeneration, we cloned several osteoclastic markers: nuclear factor of activated T cell c1 , tartrate-resistant acid phosphatase (TRAP), cathepsin K and matrix metalloprotease 9, and several osteoblastic markers: runt-related transcription factor 2 (Runx 2), osterix (OSX), type 1 collagen (COL-1), alkaline phosphatase (ALP) and osteocalcin. The expression patterns of these osteoclastic and osteoblastic markers were examined during the scale resorption and regeneration using a quantitative real-time PCR. As a result, we found that these osteoclastic markers were expressed in the multinucleated osteoclasts and increased remarkably during the scale resorption after intramuscular autotransplantation of the scale. On the other hand, these osteoblastic markers were expressed in the regenerating scale, and the expression of OSX, COL-1, ALP and osteocalcin mRNAs increased remarkably during the scale regeneration although Runx 2 mRNA did not change.

Melatonin is a secretory product of the vertebrate pineal gland which is synthesized during darkness and exhibits a conspicuous circadian rhythm. Using the cultured normal scale of goldfish, we recently demonstrated that melatonin suppressed osteoclastic activity. Then, we determined whether melatonin suppresses 1) a differentiation of single-nucleate preosteoclasts from hematopoietic stem cells and 2) an induction of multinucleated osteoclasts from the single-nucleate preosteoclasts. We cloned receptor activator of nuclear factor κB (RANK) expressed on osteoclast precursors and RANK ligand (RANKL) expressed on osteoblasts in the scale of goldfish. RANK-RANKL system plays a key role in the osteoclast differentiation. In autotransplanted scales after 1day by the treatment with melatonin, RANK and RANKL mRNA expressions were significantly lowered compared with those of autotransplanted control. In autotransplanted scales after 7days, TRAP activity significantly increased and lots of multinucleated osteoclasts appeared; however, by the treatment with melatonin from day 3 to day 7, the TRAP activity significantly decreased and lots of single-nucleate preosteoclasts were still observed.

These findings indicate for the first time that physiological concentrations of melatonin suppress the differentiation of osteoclasts and the induction of multinucleated osteoclasts *in vivo* in the teleost scale.

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