

PROCEEDINGS OF THE JAPAN SOCIETY FOR
COMPARATIVE ENDOCRINOLOGY

No.21, 2006

Abstracts of the 31st Annual Meeting and the 31st Symposium on
“Nanobioscience of Chemical Mediation:
New Applications in Comparative Endocrinology”
and
“New World of Chemical Mediation Revealed by
Single Molecule and Cell Analyses”

Sapporo
November 3-4, 2006

Edited by

S. Tanaka and M. Suzuki

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FUTURE OF NANO-BIOTECHNOLOGY: LESSON FROM HISTORY OF NEUROBIOLOGY

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Galvani L. (1737-1789) was an anatomy biologist. His contribution to physiology, neurobiology and electronic technology was remarkable. His finding of actions of nerve and muscle in 1791 gave great idea for finding a battery of Volta A. in 1780. These findings were the first contribution of biology to modern technology. Studying biology is not only to understand the lives or evolution but also to understand the design principle that exist in our world. Recently, every field of technology, mathematics, chemistry and physics etc have been developed, which accelerates developing every field of biology. However, still studying biology is not enough to understand where we were from and where we are going. In most cases, the strategy of biologists is focusing on the element of the lives to understand each interest. This might be one of the reasons why biology is developing slowly although now rather quicker than before. To overcome this situation I would like to suggest adopting modeling technique and computational simulation to investigate biology systematically. Hodgkin A.L. and Huxley A.S. (1952) built a model that demonstrates events of a neuron ⁽¹⁾. This model is excellent and accelerates to develop neurobiology in particular electrophysiology. Still after half a century, this model demonstrates many neuronal events even new findings are appeared. This strongly suggests that modeling and simulation techniques are powerful to accelerate developing neurobiology. Grillner P. has also adopted modeling and computational simulation in his research ⁽²⁾. Adopting modeling and simulation to investigate neurobiology must provide us better idea of design principle of nervous system, which in turn accelerates developing many kinds of technology.

Recent development of nanotechnology is remarkable. Nanotechnology also accelerates developing every field of biology. To understand nature of lives and common design principle of lives, it is necessary to reconstruct every hierarchy of system from elements found. Therefore, modeling and simulation techniques must be powerful tools for the future of nanobiotechnology.

(1) Hodgkin AL. and Huxley AS. (1952) *J. Physiol.*, **117**, 500-544.

(2) Grillner S. and Wallén P. (2002) *Brain Res. Rev.*, **40**, 92-106.

1: modeling, 1: computer simulation, 1: electrophysiology, 1: behavior, 1: nervous system

**STUDIES OF BIOMOLECULAR FUNCTION IN LIVING CELL NUCLEUS
USING FLUORESCENCE CORRELATION SPECTROSCOPY**

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Fluorescence correlation spectroscopy (FCS) is a unique method to determine diffusional properties and number concentrations of fluorescent molecules with single molecule sensitivity in aqueous solution by monitoring the fluctuations in fluorescence intensity in a very tiny detection area. We are applying FCS to investigation of the physiological microenvironments of subcellular compartments (organelles) in living cells. The cell nucleus contains many macromolecules that form multimolecular complexes and structures. These molecules are involved in dynamic cell activities such as transcription and molecular transportation from and to the nucleus. The local environment in the nucleus is expected to vary during different cell activities. To analyze the nuclear microenvironment in the living cell, we have constructed tandem-oligomers of green fluorescent proteins (EGFPn: EGFP₁~EGFP₅) as molecular rulers and measured the diffusion of EGFPns in living cells using fluorescence correlation spectroscopy (1).

The glucocorticoid receptor (GR) is one of transcriptional regulators that control broad physiological gene networks and pathological effects in a range of diseases, and offer excellent targets for therapeutic intervention. GR is associated with several proteins in the cytoplasm. Upon ligand binding, GR is driven into the nucleus, and regulates transactivation by association with specific genomic glucocorticoid response elements (GRE). Using FCS, we analyzed diffusion properties of green fluorescence protein-tagged GR (GR-GFP) before and after addition of ligands in the nucleus of the living cell. The diffusion of GR-GFP did not change in the cytoplasm with addition of dexamethasone (Dex), which is a transactivation agonist. On the other hand, we detected a slow-moving component in the nucleus with Dex addition as well as with RU486, which acts as an antagonist. To elucidate this slow component, we constructed a deletion mutant of the ligand-binding domain (LBD) existing in the C-terminal of GR. These results indicate that the slow-moving component is not originated by binding to GRE. Moreover, they suggest that GR can bind not only to specific cofactors but also other nonspecific binding sites in DNA because its diffusion constant is excessively large compared to that of GR associated with all cofactors currently known.

(1) Pack, C. *et al.* (2006) *Biophys J.*, **91**, 3921-3936.

2: tandem EGFP; 2molecular ruler; 2 microenvironment; 2 diffusion constant;
2 Glucocorticoid receptor

REAL-TIME IMAGING OF CORTICOSTEROID RECEPTORS IN LIVING CELLS

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Adrenal corticosteroids (cortisol in humans or corticosterone in rodents) exert numerous effects in the central nervous system that regulate the stress response, mood, learning and memory, and various neuroendocrine functions. Corticosterone (CORT) actions in the brain are mediated by two corticosteroid receptors, glucocorticoid receptor (GR) and mineralocorticoid receptor (MR), and they show a high degree of colocalization in the hippocampal region. These receptors are predominantly resided in the cytoplasm without ligand and translocated into the nucleus upon ligand binding to act as transcriptional factors. Thus their subcellular localizations are important component of their biological activity. Given the differential action of MR and GR in the central nervous system, it is important to elucidate how the trafficking of these receptors between cytoplasm and nucleus and their interaction are regulated by ligand or other molecules to exert transcriptional activity. In this symposium, we focus on the nucleo-cytoplasmic and subnuclear trafficking of GR and MR in neural cells and non-neural cells. Dynamics of MR and GR in living cultured hippocampal neurons and COS-1 cells were presented using real time imaging technique with green fluorescent protein (GFP) and its color variants including fluorescence recovery after photobleach (FRAP) (1). Effects of various kinds of effectors on the nucleo-cytoplasmic trafficking of these receptors, such as ligands, cytoskeletal elements, HSP90, and carrier proteins of importin α and β , were investigated. Furthermore, we will discuss possible heterodimerization of MR and GR by using fluorescence resonance energy transfer (FRET) in living cells (2).

(1) Nishi *et al.* (2001) *Mol. Endocrinol.*, **15**, 1077-1192.

(2) Nishi *et al.* (2004) *J. Neurosci.*, **24**, 4918-4927.

GLASS MICROCAPILLARY OSMOMETER THAT CAN MEASURE REAL TIME CHANGES IN THE INTRACELLULAR OSMOLARITY: ITS DEVELOPMENT AND APPLICATION

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A novel microcapillary osmometer that could measure the osmolality of the cytosol with a time constant of less than a second was developed. The osmometer consists of an osmotic pressure sensor probe and an electronic pressure feedback system. The sensor probe was made of a glass microcapillary with a tip diameter of $\sim 1\mu\text{m}$. The tip was plugged by semipermeable cupric ferrocyanite. An osmotic pressure reference solution was introduced into the capillary. Mineral oil was then introduced into the capillary to make a meniscus with the reference solution. When the tip of the probe was inserted into a cell, water moves across the plug according to an osmotic pressure difference between the reference solution and the cytosol, and the meniscus was thereby shifted. The meniscus shift was detected by a photo-sensor that produced an electrical signal proportional to the shift. The electrical signal was amplified and fed into a pressure generator to generate a hydraulic pressure in the probe that counteracts the water movement. The hydraulic pressure was monitored by using a pressure sensor in the probe. The osmotic pressure of the cytosol could be determined as a difference between the osmotic pressure of the reference solution and the counter pressure (1).

When the osmometer probe is inserted into a cell together with a microelectrode and a micro-ion-selective electrode, the time course of change in the cytosolic osmolality of the cell after changing the external osmolality can be determined simultaneously with changes in the membrane potential and the ionic strength of the cytosol. These physiological parameters are indispensable for understanding the mechanisms of osmoregulation.

If the semipermeable plug is removed from the osmometer probe, the osmometer can now be used as a manometer that measures the hydraulic pressure of the cytosol. Bulk modulus of the cell (2) and the amount of osmotically non-active portion of the cytosol can be determined based on the hydraulic pressure of the cytosol.

(1) Naitoh (2002) *Molecul. Biol. Cell.*, Suppl., **13**, 223a.

(2) Iwamoto *et al.* (2005) *J. Exp. Biol.*, **208**, 523-537.

SI-4: microcapillary osmometer, osmotic pressure, cytosolic pressure, osmoregulation, bulk modulus.

SI-5

AGGRESSIVE BEHAVIOR REGULATION IN A JAPANESE CARPENTER ANT

Mamiko Ozaki

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The struggle to maintain order in societies has led social animals including human beings to evolve and developed various means of communication. Ants have developed a sophisticated chemical communication system that enables them to reject non-nestmate conspecifics and to accept nestmates. Our goal is to understand the underlying mechanisms involved in these critical decisions. Elucidation of this problem has been elusive and subject to much speculation, especially at the neurophysiological level. Our study addresses this problem and suggests a novel stepwise mechanism from the peripheral to the central nervous systems via a newly discovered sensory organ that acts to filter pheromonal information(1).

The newly discovered sensory organ on the antennae of *Camponotus japonicus* looked like an olfactory sensillum with multiporous surface, but is actually sensitive to contact stimulation with cuticular hydrocarbon (CHC) blends (Here we certified that the CHCs are pheromonal chemical cues for aggressive behavior of the ant.) of non-nestmates but not nestmates. Thus, it functions as a nestmate-information-cut filter to higher neuronal systems. Considering adaptation properties in other sensory systems, visual cells for example, we tried to discuss on presumable mechanisms for such a particular filtering function of this sensory organ.

Considering behavioral data on aggression correlated to the activities of this organ, we found regulative effect of octopamine on aggressiveness of the ants; octopamine increases the threshold of aggressive behavior. We constructed ant-octopamine receptor antiserum and attempted to found the histological localization of the octopamine receptor around the mushroom bodies and antennal lobes.

(1) Ozaki *et al.* (2005) *Science*, **309**, 311-314.

SI-5: aggressive behavior, pheromone, nestmate recognition, octopamine, ant

RECENT ADVANCES OF ETHOLOGICAL PHYSIOLOGY FOR FREE-LIVING ANIMALS BY MICRO-DEVICES

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Recently there has been remarkable progress in the development of sensory system coupled with loggers that have been attached to free-living animals, which have named bio-logging devices. Increases in sensor resolution, sensor diversity and memory size have been coupled with decreases in unit size. We have investigated diving behavior and physiology of penguins by bio-logging devices. It is well observed that a group of Adélie penguins synchronize the timing of diving and of surfacing. We employed the loggers with time depth recorder and still camera system to study underwater group behavior of Adélie penguins and revealed that several birds descended and ascended synchronously and gathered at the bottom depth to feed preys (1). Dive for foraging is essentially important for penguins. However it is still little known how penguins make it possible to perform deep and prolonged dives. To investigate air usage of penguins during dives, we evaluated the air volume of diving penguins using a newly developed logger to measure acceleration. When comparing the passive ascents for shallow and deep dives, there was a positive correlation between air volume and the depth of the dive. It was suggested that penguins regulated their air volume to optimize the costs and benefits of buoyancy (2). In case of deep dive, the time budget of diving might be modulated by the blood concentration of carbon dioxide. Further, it was suggested that penguins decreased metabolic rate and consumed oxygen in air sac during dive to perform prolonged dives by newly developed bio-logging devices equipped with oxygen electrode (3). Bio-logging will play a more important role to understand animals in extreme environment.

(1) Takahashi *et al.* (2004) *J. Ethol.*, **22**, 5-11.

(2) Sato *et al.* (2002) *J. Exp. Biol.*, **205**, 1189-1197.

(3) Stockard *et al.* (2005) *J. Exp. Biol.*, **208**, 2973-2980.

SI-6: Bio-logging, dive, penguin, data logger

SII-1

GENE EXPRESSION IN A SINGLE NEURON

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Gene expression is differently regulated in every cell even though the cells are included in the same tissue. We therefore developed a procedure for isolating a single, identifiable cell and determining the exact copy numbers of mRNAs within it (1). We first isolated the cerebral giant cell of the pond snail *Lymnaea stagnalis* as this neuron plays a key role in the process of memory consolidation of a feeding behavior. We then determined the copy numbers of mRNAs for the cyclic AMP-responsive element binding proteins (CREBs). The protocol uses two techniques in concert with each other: a technique for isolating a single neuron with newly developed micromanipulators coupled to an assay of mRNAs by quantitative real-time reverse transcription-polymerase chain reaction (real-time PCR). The molecular assay determined the mRNA copy numbers, each of which was compared to a standard curve prepared from cDNA solutions corresponding to the serially diluted solutions of *Lymnaea* CREB mRNA. The standard curves were linear within a range between 10 to 10⁵ copies, and the intra-assay variation was within 15%. Each neuron removed from the ganglia was punctured to extract the total RNA directly and was used for the assay without further purification. Using this two-step procedure, we found that the mRNA copy number of CREB repressor (CREB2) was 30-240 in a single cerebral giant cell, whereas that of CREB activator (CREB1) was below the detection limits of the assay (< 25). These results suggest that the CREB cascade is regulated by an excess amount of CREB2 in the cerebral giant cells. Next, the amounts of CREB1 and CREB2 mRNAs were changed by an RNA interference method. We thus injected the small interfering RNA (siRNA) of CREB1 or CREB2 into the cerebral giant cells, and then examined the changes in amplitude of excitatory postsynaptic potential (EPSP) recorded in the B1 motoneurons (2). The EPSP amplitude was suppressed 15 min after injection of CREB1 siRNA, whereas that was augmented 60 min after injection of CREB2 siRNA.

(1) Wagatsuma *et al.* (2005) *J. Exp. Biol.*, **208**, 2389-2398.

(2) Wagatsuma *et al.* (2006) *J. Neurosci. Res.*, **84**, 954-960.

SDII-1: CREB, mRNA, real-time PCR, RNAi

SII-2

IDENTIFICATION OF NOVEL G-PROTEIN COUPLED RECEPTORS IN LASER-CAPTURED SINGLE GnRH NEURONS

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We have developed a novel single cell real-time quantitative PCR technique, which incorporates harvesting marker-identified single cells using laser-capture. Here, for the first time in a vertebrate species, using this innovative single cell gene profiling technique, we report the presence of G-protein coupled receptors in individual gonadotropin-releasing hormone (GnRH) neurons and endocrine cells of the pituitary of the tilapia *Oreochromis niloticus*.

The differential expression of multiple combinations of three GnRH receptor types (R1, R2 and R3) in individual gonadotropic and nongonadotropic cells demonstrates cellular and functional heterogeneity. The differential use of GnRH receptors in corticotropes, melanotropes and thyrotropes during gonadal maturation and reproductive behaviors suggests new roles for these hormones.

Further, we provide evidence of the structure of a novel nonmammalian G-protein coupled receptor (GPR54) for kisspeptins, encoded by Kiss-1 gene, which is highly conserved during evolution and expressed in GnRH1, GnRH2 and GnRH3 neurons. We hypothesize GPR54 stimulates GnRH secretion and is crucial for pubertal maturation. We speculate, the use of this method will allow the identification and quantification of known and unknown genes in single cells, which would greatly facilitate our understanding of the complex interactions that govern the physiology of individual cells in vertebrate species.

SII-2: GnRH, G-protein coupled receptor, single-neuron, puberty, neuroendocrinology

SII-3

CHROMATIN REGULATION AT THE HUMAN GROWTH HORMONE GENE CLUSTER

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The human growth hormone (*hGH*) gene cluster, located on chromosome 17q22-24, is composed of five structurally conserved genes: *hGH-N*, chorionic somatomammotropin-like (*hCS*)-*L*, *hCS-A*, *hGH-variant* (*hGH-V*), and *hCS-B*. Although the primary structures are highly conserved among those five genes, they show mutually exclusive tissue specificities. *hGH-N* is expressed only in anterior pituitary whereas expression of the other four genes is restricted to the syncytiotrophoblast (STB) layer of placental villi. Our previous data have demonstrated that the activation of the *hGH* cluster in the two tissues is dependent on a set of remote locus control region (LCR) including five DNase I hypersensitive sites (HS) located between 15 and 32 kb 5' to the cluster. The HSI at -15 kb plays key roles for establishing a large histone acetylation domain and the LCR domain of transcription required to activate the *hGH-N* gene in pituitary. In contrast, the molecular mechanisms for activation of the placental genes are not clear.

In order to gain insight into the placental gene activation, we investigated epigenetic modifications at the *hGH* locus using several placental model systems. In human term placental STB, which express the cluster genes at maximum levels, we identified high levels of histone acetylation and methylation at the LCR and within the cluster region encompassing the four placental genes. In the BeWo cell line, which barely expressed the placental genes, only histone H3K4 methylation domains were observed. In freshly prepared cytotrophoblast (CTB) cells, discrete sites of the LCR and the cluster region were methylated at histone H3K4 and no histone acetylation was detected. During the spontaneous differentiation of the CTB to STB, the histone methylation domains were completely established and the histone acetylation levels increased at the LCR and within the cluster region. These data suggest that activation of the *hGH* cluster in the placenta is dependent on a series of epigenetic modifications and that histone acetylation and methylation have different and dynamic roles in this pathway.

SII-3: histone modification, placenta, differentiation, growth hormone, locus control region

SII-4

DYNAMICS AND MODIFICATION OF HISTONES IN LIVING CELLS

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Eukaryotic nuclear DNA is wrapped around a protein octamer composed of the core histones H2A, H2B, H3, and H4, forming nucleosomes as the fundamental units of chromatin. These core histones are highly conserved from yeast to mammals, and play key roles in chromatin function, such as transcription, DNA replication, repair, and chromosome segregation, through the regulation by post-transcriptional modification and reorganization of nucleosome structure.

We have studied the kinetics of core histones in living cells by photobleaching analysis and cell fusion, and revealed that H2B in euchromatin is exchanged more rapidly compared to H3.1 and H4 (1, 2). To understand the molecular mechanisms of histone exchange found in living cells, we developed an in vitro system using permeabilized cells, allowing the assembly and exchange of histones in situ. H2A and H2B, each tagged with the green fluorescent protein (GFP), are incorporated into euchromatin by exchange independently of DNA replication, and H3.1-GFP is assembled into replicated chromatin. By fractionating cellular factors assisting the incorporation of H2A-H2B into permeabilized-cell chromatin, we identified protein phosphatase 2C gamma-subtype (PP2C γ) as a histone chaperone that binds to and dephosphorylates H2A-H2B, in addition to nucleosome assembly protein 1 and 2. These data provide a novel link between histone deposition and dephosphorylation through a protein phosphatase, further emphasizing the importance of histone modification.

Towards understanding histone modification signals in living cells, we are generating specific antibodies against modified histones.

(1) Kimura and Cook (2001) *J. Cell Biol.*, **153**, 1341-1353.

(2) Kimura (2005) *DNA Repair*, **4**, 939-950.

(3) Kimura *et al.* (2006) *J. Cell Biol.*, **175**, 389-400.

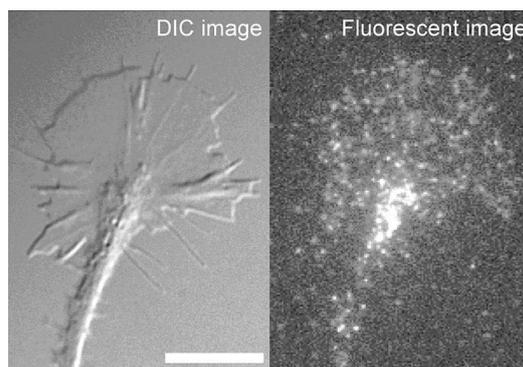
SII-4: chromatin/gene expression/living cell/nucleosome/protein phosphatase

SINGLE-MOLECULE ANALYSES OF NEUROTROPHIN SIGNALLING IN THE GROWTH CONES OF SENSORY NEURONS DURING THE ACTIVATION OF AXONAL OUTGROWTH

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Imaging of fluorescently labeled biomolecules has been used to observe in real time the interactions between ligands and receptors on various kinds of living cells. This technique allows us to analyze the local signal transduction and amplification processes in confined sub-cellular domains of neurons such as axons, dendrites, synapses and cell bodies. We employ this method to reveal the mechanisms by which neural axons and dendrites detect and reach the target tissues during development. The growth cone is a motile structure located at the distal tip of the nerve fiber.



DIC (left) and fluorescent (right) micrographs of the growth cone after the application of fluorescent NGF. Bar; 10 μ m

During embryogenesis, the growth cone serves to guide the nerve fiber to an appropriate target through the aid of guidance cues and neurotrophins. The 2.5S nerve growth factor (NGF), the founding member of neurotrophins, promotes the axonal growth of sensory and sympathetic neurons. We observed the interaction of NGF with receptors on the growth cone of chick sensory neurons using a novel fluorescent conjugate of NGF, Cy3-NGF. Upon application of 400 pM of fluorescent-NGF, the growth cones responded within one minute of adding the stimulus by expanding their lamellipodia. Imaging of fluorescent NGF enabled us to analyze the *in situ* kinetics of NGF and the receptor interaction on the growth cone. Based on these analyses, the estimated number of high affinity receptors on a single growth cone was approximately 200 and that of low affinity receptors was 700. Surprisingly, only 40 molecules of bound NGF, which occupied less than 5% of the estimated total binding sites on a single growth cone, could initiate the motile responses of a single growth cone. After binding to the high-affinity receptor, Cy3-NGF displayed lateral diffusion, which was followed by a one-directional rearward movement toward the central region of the growth cone. The one-directional movement of Cy3-NGF displayed the same rate as the rearward flow of actin. Molecules of Cy3-NGF were internalized in the vicinity of the central region of the growth cone during this rearward trafficking. These results suggested that actin-driven trafficking of the NGF-receptor complex is an essential step to the accumulation and endocytosis of NGF at the growth cone. We are currently combining this single molecule imaging of neurotrophins with fluorescent protein-based imaging technology for simultaneous observations of activated receptors as well as intracellular signaling molecules.

SII-5: Single molecule imaging, nerve growth factor, TrkA, growth cone, dorsal root ganglion

SII-6

SINGLE-NEURON MOLECULAR PHYSIOLOGICAL ANALYSIS OF THE MULTIFUNCTIONAL GnRH PEPTIDERGIC SYSTEMS

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The GnRH peptidergic neuron systems consist of hypothalamic neuroendocrine and extrahypothalamic neuromodulatory GnRH neurons. Here, I introduce our recent studies on the physiological properties of neuroendocrine preoptic (POA) GnRH neurons and the neuromodulatory terminal nerve (TN-) and tegmental (TEG-) GnRH neurons, and their control by metastin peptidergic neuron system¹. We use a fish model system in which we can easily identify all of them in intact or GnRH-GFP transgenic fish brain preparations *in vitro*, which is advantageous over brains of most other vertebrates for the single-neuron molecular physiological analysis of multifunctional GnRH peptidergic systems. In contrast to the regular pacemaker activities of TN- and TEG-GnRH neurons, the POA-GnRH neurons showed alternating active and silent phases of firing activities. Now that we have various single-neuron molecular physiological experimental approaches (electrochemical measurement of GnRH release, Ca²⁺ imaging after single-cell electroporation, single-cell RT-PCR, double patch clamp recording, etc.) and experimental systems (*in vitro* preparations of the dwarf gourami and three lines of GnRH-GFP transgenic medaka) in hand, simultaneous multidisciplinary approaches can be applied to study the physiology of multifunctional GnRH neurons. We also describe our recent studies on a new metastin peptidergic neuronal system. We found that the metastin-ir cell bodies are distributed in two distinctive brain regions. Double immunocytochemistry showed that metastin-ir fibers project not only near the POA-GnRH neurons but also near TEG- and TN-GnRH neurons. Furthermore, the metastin neurons project to wide areas in the brain (mainly sensory-related brain areas), suggesting that it may modulate the sensory information processing, while it stimulates LH surge.

- (1) Abe, H. and Oka, Y. (2007) Neuromodulatory functions of terminal nerve-GnRH neurons. In : *Fish Physiology* Vol. 25, Sensory Systems Neuroscience, pp. 455-503.

SII-6: GnRH, physiology, imaging, single-neuron, neuroendocrinology

MOLECULAR CLONING AND GENE EXPRESSION PATTERN OF ECDYSONE 20-MONOOXYGENASE IN THE SILKWORM, *Bombyx mori*

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In silkworm, *Bombyx mori*, it is known that 20-hydroxyecdysone (20E) is indispensable for embryonic development (1). 20E was demonstrated to be formed by both dephosphorylation of physiologically inactive ecdysteroid-phosphates and *de novo* synthesis. The hydroxylation of ecdysone (E) at the C-20 position, which is catalyzed by ecdysone 20-monooxygenase (E20OHase), is a rate-limiting step in the *de novo* synthesis (2). In this study, a cDNA sharing a high sequence similarity with known E20OHases was isolated from eggs by using 3' and 5'-RACEs (Maeda *et al.*, 2005, Gene bank accession No.: 236417). Deduced amino acid sequence of obtained clone has 48% and 81% identity with *Drosophila melanogaster* and *Munduca sexta* E20OHases, respectively. We assume that this clone is *B. mori* E20OHase by using phylogenetic analysis. To confirm the enzyme activity of the cloned *B. mori* E20OHase, the recombinant E20OHase was expressed using baculovirus expression system. Sf9 cells were infected with recombinant baculovirus containing the whole open reading frame of *B. mori* E20OHase. The infected cell extract was incubated with E, and the product was analyzed using reverse-phase HPLC. HPLC profile demonstrated that the product is 20E. Semi-quantitative reverse transcription-PCR analysis revealed that E20OHase mRNA levels increased 2 days after oviposition and reached a peak 3-4 days after oviposition, and then decreased gradually to the basal level in nondiapauses eggs. In diapauses eggs, E20OHase mRNA remained at a low level. This result coincided with the pattern of changes in E20OHase activity (3), suggesting that the gene transcription is required for the induction of E20OHase activity.

(1) Makka *et al.* (2002) *Arch. Insect Biochem. Physiol.*, **51**, 111-120.

(2) Sonobe *et al.* (1999) *Zool. Sci.*, **16**, 935-943

(3) Horike and Sonobe (1999) *Arch. Insect Biochem. Physiol.*, **41**, 9-17.

P1: keyword: silkworm, ecdysone, P450, diapause, monooxygenase

CLONING AND EXPRESSION OF GENES ENCODING FOUR DIFFERENT SUBTYPES OF MELATONIN RECEPTORS IN GOLDFISH

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Melatonin is synthesized primarily in pineal gland and retina, where its synthesis and release exhibit a diurnal rhythm with peak activity during the dark period. Melatonin is involved in multiple physiological functions including circadian rhythms and seasonal reproduction. Melatonin receptors (Mel) belong to the G-protein coupled receptor superfamily. In vertebrates, there are three types of Mels, termed as Mel1a (MT1), Mel1b (MT2), and Mel1c. In teleosts, two different subtypes of Mel1a were identified, and termed as Mel1a1.4, and Mel1a1.7. Mel1b and Mel1c were termed as Mel1b2.6 and Mel1c2.3, respectively. The distribution of Mel1a1.4 and Mel1a1.7 transcripts has been reported mainly in the brain and pituitary (1), while that of Mel1b2.6 transcript has been reported in the brain and various peripheral organs, such as retina, liver, spleen, heart, and kidney (2). However, the distribution of Mel1c2.3 transcript has never been reported yet. As a first step to clarify molecular mechanisms of action of fish Mels, we determined partial sequences of the four subtypes of Mel genes in goldfish, and tissue distribution of these transcripts were examined by reverse transcription (RT)-PCR.

Four partial DNA fragments were amplified from genomic DNA by PCR using primers that correspond to the transmembrane domain 2 and 7 of zebrafish Mel genes. They were cloned into a pCR-Script plasmid vector and their sequences were determined. The similarities of the deduced amino acid sequences between goldfish and zebrafish were 96-100%. Total RNA was extracted from the brain, pituitary, eyeball, gill, atrium, bulbus arteriosus, intestine, spleen, kidney, and scale of goldfish. RT-PCR was carried out using four sets of specific primers. The transcripts of Mel1a1.4 and Mel1a1.7 were distributed in the brain, gill, bulbus arteriosus, spleen, and kidney. Mel1a1.7 transcript was also detected in the eyeball. Mel1b2.6 transcript was detected in all tissues examined. Mel1c2.3 transcripts were detected only in the kidney. These results indicate that four subtypes of Mel genes are differentially expressed in the examined tissues of goldfish.

(1) Qiong *et al.* (2004) *Gen. Comp. Endocrinol.*, **136**, 311-321.

(2) Park *et al.* (2006) *J. Pineal Res.*, **41**, 166-174.

P2: Melatonin, Melatonin receptor, Goldfish, Gene expression

CLONING OF THE cDNAs ENCODING BONE MORPHOGENETIC PROTEINS FROM RAINBOW TROUT

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The thyroid hormones play important roles in the regulation of development, metabolism, homeostasis, and reproduction. These hormones are synthesized in the thyroid follicles, which are scattered over the ventral aorta in the rainbow trout. To understand the molecular mechanisms underlying the development of the thyroid, we previously carried out histological analysis of thyroidogenesis in the rainbow trout, using Nkx2.1 and Pax8 as molecular markers. As a result, *in situ* hybridization histochemistry of trout embryos showed the thyroid primordium to be surrounded by larger blood vessels, suggesting that paracrine factors, secreted from the blood vessels, might be involved in thyroidogenesis in the trout. In mice, bone morphogenetic protein (BMP) 4 is known to be expressed in the outflow-tract myocardium of the developing heart (1). BMPs can also induce formation of adipose tissue and cartilage, both of which are observed around the thyroid (2). In the present study, we have identified partial sequences of three distinct BMP cDNAs from a ZAP Express cDNA library prepared from the ventral aorta with the thyroid of the adult trout. PCR was performed using degenerate primers, and sequence analysis of the amplified products revealed the partial sequences of three types of cDNAs encoding rainbow trout BMPs, rtBMP2/4, rtBMP5/6, and rtBMP7. The rtBMP2/4 cDNA is composed of 150 bp, and the deduced amino acid sequence has 78% and 75% similarity to mouse BMP2 and BMP4, respectively. The rtBMP5/6 cDNA is composed of 64 bp, and the predicted amino acid sequence shows 74% and 78% similarity to mouse BMP5 and BMP6, respectively. The rtBMP7 cDNA is composed of 64 bp, and the predicted amino acid sequence exhibits 86% similarity to mouse BMP7. Because these clones were obtained from the ventral aorta with the thyroid, it is possible that BMPs might have some effects on thyroid gland in the rainbow trout.

(1) Liu, W. *et al.* (2004) *Proc. Natl. Acad. Sci.*, **101**, 4489-4494.

(2) Rebbapragada, A. *et al.* (2003) *Mol. Cell. Biol.*, **23**, 7230-7242.

Keywords: Thyroid, BMP, Pax8, Nkx2.1, rainbow trout

CLONING OF THE RECEPTOR FOR FROG GROWTH HORMONE-RELEASING PEPTIDE IN THE BRAIN OF BULLFROGS, *Rana catesbeiana*

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We previously identified a novel hypothalamic neuropeptide in the brain of bullfrogs. This peptide stimulated the release of growth hormone (GH) from the pituitary of bullfrogs and therefore was named frog GH-releasing peptide (fGRP) (1). The precursor cDNA encoded fGRP and three fGRP-related peptides (fGRP-RPs; fGRP-RP-1, -RP-2 and -RP-3) that shared the same C-terminal motif, Leu-Pro-Xaa-Arg-Phe-NH₂ (Xaa=Leu or Gln; LPXRFamide peptides) (2). These related peptides were also identified as endogenous ligands in the bullfrog brain (3). Interestingly, fGRP-RPs have various functions such as pain modification (fGRP-RP-1) (4), stimulation of GH release (fGRP-RP-2) (3) and stimulation of prolactin release (fGRP-RP-2) (3). Identification of the receptor for fGRP is crucial to elucidate the mode of action of fGRP. We therefore cloned a putative receptor for fGRP in the bullfrog brain. We first amplified a partial fragment of the putative fGRP receptor using PCR primers designed from the sequence of the receptor of other LPXRFamide peptides. Then we amplified the 5' end and 3' end of the fragment by a combination of 3' and 5' rapid amplification of cDNA ends (RACE) using PCR primers specific for the partial sequence of the putative fGRP receptor. The nucleotide and deduced amino acid sequences of the putative fGRP receptor revealed a full length of 1503 bp and an open reading frame of 1299 bp encoding 433 amino acid residues with a calculated molecular mass of 49.6 kDa. Hydrophobic analysis revealed that the putative fGRP receptor possessed seven transmembrane domains, indicating a new member of the G protein-coupled receptor superfamily.

(1) Koda, A. *et al.* (2002) *Endocrinology*, **143**, 411-419.

(2) Sawada, K. *et al.* (2002) *J. Endocrinol.*, **269**, 6000-6008.

(3) Ukena, K. *et al.* (2003) *Endocrinology*, **144**, 3879-3884.

(4) Kanetoh, T. *et al.* (2003) *Comp. Biochem. Physiol.*, **134**, 259-266.

P4: LPXRFamide peptide, fGRP, GPCR, cloning

EXPRESSION OF NOVEL ISOFORM OF BULLFROG PROLACTIN RECEPTORItaru Hasunuma¹, Kazutoshi Yamamoto¹ and Sakae Kikuyama^{1,2}¹Department of Biology, School of Education, Waseda University, Tokyo 169-8050, Japan.²Department of Biology, Faculty of Science, Shizuoka University, Shizuoka 422-8529, Japan.

It is known that prolactin receptor (PRLR) belongs to class I cytokine receptor superfamily. In mammalian species, existence of several isoforms of PRLR such as long, intermediate, and short forms has been reported (1). Previously, we obtained bullfrog PRLR cDNA encoding 617 amino acids that exhibited the feature of mammalian long form PRLR (2). In the present study, a novel isoform cDNA of bullfrog PRLR was cloned by the 3'-RACE method using bullfrog kidney cDNA as a template. This isoform cDNA encoded 242 amino acids. The sequence of extracellular domain of the isoform (from translation initiation Met to Gly³²¹) was completely identical with bullfrog long form PRLR, but the transmembrane domain and intracellular domain were lacking. In the C-terminus, it contained an isoform-specific amino acid sequence comprising 11 amino acids (NDLIYITHHII). This novel PRLR isoform structure was similar to PRL-binding protein identified in mammalian species (3). Expression of mRNA of this isoform was observed in the brain, lung, liver, kidney, testis, and ventral and dorsal skin of adult frog. To confirm that this isoform binds PRL, PRL-binding assay was performed. A cDNA fragment containing open reading frame was obtained by RT-PCR, and ligated into pCS2+ mammalian expression vector. Transient expression assays were conducted by using COS-7 monkey kidney cells. Cells were harvested at 48 h after the transfection, and then scraped, lysed by four repeated freeze-thaw cycles. Specific binding of ¹²⁵I-labeled bullfrog PRL to water-soluble protein fraction of cell lysate was observed. Furthermore, the specific binding activity was also detected in the medium in which COS-7 cells were transiently transfected with a construct containing the isoform cDNA were harvested. The result suggests that novel PRLR isoform protein was secreted from COS-7 cells to the culture medium.

(1) Bole-Feysot *et al.* (1998) *Endocrine Rev.*, **19**, 225-268.

(2) Hasunuma *et al.* (2004) *Gen. Comp. Endocrinol.*, **138**, 200-210.

(3) Trott *et al.* (2003) *J. Mol. Endocrinol.*, **30**, 31-47.

P5: prolactin, prolactin receptor, isoform, bullfrog

ANALYSIS OF mRNAs EXPRESSED IN THE PARATHYROID GLAND OF THE BULLFROG

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The parathormone (PTH), derived from the parathyroid gland, is of prime importance for the control of calcium homeostasis in mammals, and well known to increase circulating levels of calcium. Phylogenetically, the parathyroid first occurs in amphibians, but PTH gene already exists in fish genome. In zebrafish embryogenesis, PTH is found in lateral line neuromasts (1), whereas glial cells missing 2 (*Gcm2*), a marker gene for the parathyroid, is expressed in the pharyngeal epithelium and gill filament buds (2). For the understanding of the molecular mechanisms underlying the occurrence of the parathyroid in vertebrates, it is indispensable to identify more molecules specific to this endocrine gland, and further to investigate the cellular localization and function of these molecules. In the present study, we have examined mRNAs expressed in the parathyroid of the bullfrog, *Rana catesbeiana*, using molecular cloning and suppression subtractive hybridization (SSH). By RT-PCR and subsequent sequence analysis of the amplified products, we first tried identifying GCM2 mRNA to confirm that the collected organ was the parathyroid. As a result, not only GCM2 cDNA but also GCM1 cDNA was cloned partially. The partial amino acid sequences of bullfrog GCM1 and GCM2 showed 82% and 89% similarity to *Xenopus* GCM1 and GCM2, respectively. Although only *Gcm2* is detected in mammalian parathyroid, both *Gcm1* and *Gcm2* seem to be expressed in the bullfrog parathyroid. The SSH analysis between the parathyroid and fat body further revealed the expression of transcripts similar to transposase/inhibitor of apoptosis protein 1, reverse transcriptase, transport permease, elongation factor 1, *etc.*, in the parathyroid. 10 novel transcripts were also isolated. Among these transcripts, however, PTH mRNA could not be identified presumably because the amino acid sequence of PTH is not highly conserved between vertebrate classes. It is also possible that PTH might not be produced abundantly in bullfrog parathyroid.

(1) Hogan, B.M. *et al.* (2005) *Endocrinology*, **146**, 547-551.

(2) Hogan, B.M. *et al.* (2004) *Dev. Biol.*, **276**, 508-522.

Keywords: parathyroid hormone, GCM1, GCM2, suppression subtractive hybridization, bullfrog

TISSUE DISTRIBUTION AND DEVELOPMENT OF CHICKEN MOTILIN CELLS

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It is well established that motilin, a 22-amino-acid peptide, is mainly produced in the mucosal layer of the mammalian duodenum. Although the sequence and structure of chicken motilin have been determined, the tissue distribution and morphological characteristics of motilin-producing cells (motilin cells) in the chicken gastrointestinal tract are still unknown. In this study, we investigated motilin expression and distribution of motilin cells in the central and peripheral tissues of hatching (3 days) and adult (125 days) chickens by RT-PCR and *in situ* hybridization (ISH).

The results of RT-PCR showed that motilin mRNA was slightly expressed in the hypothalamus, cerebellum and pituitary gland and was abundantly expressed in the medulla oblongata, duodenum and large intestine of the hatching chicken. Although motilin mRNA expression was also found in the duodenum and ileum in the adult chicken, the expression levels of motilin mRNA were lower than these in the hatching chicken. In the medulla oblongata, motilin mRNA was transiently expressed at the hatching stage but was not observed at any other stages.

ISH analysis revealed that motilin mRNA-expressing cells (motilin-ex cells) in the hatching chicken were distributed in the mucosal layer but not in the myenteric plexus in the duodenum and existed only as closed-type cells. On the other hand, no motilin-ex cells were found in the adult chicken duodenum. These results indicate that motilin mRNA expression level in the chicken duodenum gradually decreases during development and suggest that motilin in the medulla oblongata plays an important role in some functions of the central nervous system in the hatching chicken.

P7: Motilin, Chicken, *in situ* hybridization

cDNA CLONING AND EXPRESSION PROFILE OF mRNA FOR CHICKEN GPR39

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GPR39 belongs to the subfamily of G protein-coupled receptors containing 7 transmembrane domains, and shows high homology to ghrelin receptor, GHSR (1). GPR39 has recently been characterized as a specific receptor for a novel anorexic peptide hormone, obestatin, isolated from rat stomach (2). However, negative results for the anorexic action and GPR39 binding of obestatin have also been reported. In this study, we performed cDNA cloning for chicken GPR39 and characterized expression profiles of its mRNA in chicken tissues. Total RNA was extracted from chicken tissues and GPR39 cDNA was cloned by RT-PCR using primers designed from the putative GPR39 gene sequence appeared in chicken genome database. The cDNAs for 3'- and 5'- regions were cloned by RACE. The cloned GPR39 cDNA was about 3.0 kb in length and encoded 462 amino acids. The amino acid sequence showed significant homology to mammals GPR39 (>60%). Realtime PCR analysis for GPR39 mRNA expression was carried out using primers derived from Exon 1 (sense) and Exon 2 (antisense) of GPR39 gene. Realtime PCR analysis revealed that chicken GPR39 mRNA was expressed in all the tissues examined with high levels in digestive organs and oviduct. The expression level of GPR39 in duodenum was rapidly increased during post-hatch period. These findings suggest that GPR39 might be involved in peristaltic motion in digestive and reproductive ducts in chicken.

(1) McKee *et al.* (1997) *Genomics*, **46**, 426-434.

(2) Zhang *et al.* (2005) *Science*, **310**, 996-999.

P8: Chicken, GPR39, Obestatin, Duodenum, Oviduct

GONADOTROPES EXPRESS THE RECEPTOR FOR GONADOTROPIN-INHIBITORY HORMONE (GNIH) IN QUAIL

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Recently, we identified a novel hypothalamic dodecapeptide, Ser-Ile-Lys-Pro-Ser-Ala-Tyr-Leu-Pro-Leu-Arg-Phe-NH₂, in the brain of Japanese quail (*Coturnix japonica*) (1). This avian neuropeptide was shown to inhibit gonadotropin release from the cultured quail anterior pituitary (1). We therefore termed it gonadotropin-inhibitory hormone (GnIH) (1). This is the first hypothalamic peptide inhibiting gonadotropin release reported in any vertebrate. GnIH was also effective in inhibiting LH *in vivo* (2). Interestingly, chronic treatment with GnIH inhibited gonadal development and maintenance by inhibiting gonadotropin release and synthesis in quail (2). Because GnIH was shown to be located in neurons of the paraventricular nucleus and their terminals in the median eminence (1, 3, 4), GnIH may act directly on the pituitary to inhibit gonadotropin release and synthesis. To elucidate the mode of action of GnIH, we then identified a novel G protein-coupled receptor for GnIH in quail (5). Southern blotting analysis of reverse-transcriptase mediated PCR products revealed the expression of GnIH receptor mRNA in the quail pituitary (5). In this study, we characterized the localization of GnIH receptor in the quail pituitary by *in situ* hybridization of GnIH receptor combined with immunocytochemistry for LH. GnIH receptor was located in gonadotropes in the quail pituitary. Thus, it appears that GnIH acts directly on gonadotropes via GnIH receptor to inhibit gonadotropin release and synthesis.

(1) Tsutsui, K. *et al.* (2000) *Biochem. Biophys. Res. Commun.*, **275**, 661-667.

(2) T. Ubuka, T. *et al.* (2006) *Endocrinology*, **147**, 1187-1194.

(3) K. Ukena, K. *et al.* (2003) *Cell Tissue Res.*, **312**, 73-79.

(4) Ubuka, T. *et al.* (2003) *J. Endocrinol.*, **178**, 311-318.

(5) Yin, H. *et al.* (2005) *J. Endocrinol.*, **184**, 257-266.

P9: GnIH, GnIH receptor, Gonadotropes, Quail pituitary.

EXPRESSION OF RETINALDEHYDE DEHYDROGENASE (RALDH) IN THE EMBRYONIC ANTERIOR PITUITARY GLANDS OF RATS

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Retinoic acid (RA) is a signaling molecule essential for both normal embryonic development and tissue differentiation in vertebrates. RA is also thought to act as a differentiation factor in pituitary cells. RA receptors are expressed in the mouse embryonic pituitary gland (1). RA acts on non-hormone-producing cells among pituitary cells and induces growth hormone-producing cell differentiation *in vitro* (2). RA was suggested to induce pituitary development. However, whether RA is generated in the pituitary gland and plays a role as a paracrine and/or autocrine hormone is generally unknown. RA is synthesized from retinoids through oxidation processes. Dehydrogenases that catalyze oxidation of retinal to RA are members of the retinaldehyde dehydrogenase (RALDH) family. In this study, we examined the expression of RALDH1, RALDH2, and RALDH3 mRNA in the rat embryonic pituitary gland. Using *in situ* hybridization with digoxigenin-labeled cRNA probes, we detected mRNA expression of RALDH2 and RALDH3 but not RALDH1 in Rathke's pouch and later, in the developing anterior pituitary gland. The RALDH2 and RALDH3 mRNA signals were faint in the anterior pituitary gland of neonates. At E14.5, neither RALDH2 nor RALDH3 was expressed in cells positive for expression of α GSU mRNA. Our findings suggest that RALDH2 and RALDH3 are expressed in undifferentiated developing pituitary cells. We also used quantitative real-time PCR to analyze RALDH2 and RALDH3 mRNA expression levels during development of the pituitary gland. As expected, RALDH2 and RALDH3 mRNA levels in the pituitary gland gradually decreased through the embryonic stages to the postnatal stages. These results suggest that RA is generated by RALDH2 and RALDH3 in the developing anterior pituitary glands of rats. RA may play a role as a paracrine and/or autocrine signaling molecule in the developing anterior pituitary gland.

(1) Dolle. *et al.* (1990) *Development*, **110**, 1133-1151.

(2) Mogi. *et al.* (2005) *J. Endocrinol.*, **184**, 41-50.

P10: Retinoic acid, Rat, Pituitary, Development, In situ hybridization

INVERSE RELATIONSHIP BETWEEN GHRELIN AND LEPTIN mRNA EXPRESSION LEVELS IN THE FASTED RAT STOMACH

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Ghrelin, an endogenous ligand for the growth hormone secretagogue receptor, is a peptide produced predominantly in the stomach (1). It has been reported that endogenous ghrelin levels are increased by fasting and decreased after re-feeding (2). It has also been reported that estrogen upregulates ghrelin expression and production (3) and that somatostatin inhibits ghrelin secretion (4), whereas leptin has a paradoxical effect (5,6). Recently, several studies have shown that estrogen, somatostatin and leptin are produced in the stomach. However, the direct effects of these gastric hormones on ghrelin expression in a fasting state remain obscure. In this study, we examined the mRNA expression levels of ghrelin, aromatase (estrogen synthetase), leptin and somatostatin in the fasted male rat stomach by quantitative RT-PCR. Then incubation of stomach tissue was carried out to analyze the direct effect of leptin on in vitro ghrelin mRNA expression. Finally, double staining of the male rat stomach for leptin and ghrelin was also performed. After 48 hrs of fasting, although gastric ghrelin mRNA expression level was significantly increased, leptin mRNA expression level was decreased. On the other hand, expression levels of aromatase and somatostatin mRNA were not changed after fasting. In vitro ghrelin mRNA expression was significantly inhibited by leptin at a dose of 100 nM. Double staining showed that leptin mRNA-expressing cells were adjacent to ghrelin-immunopositive cells. The results suggest that an elevated gastric ghrelin expression level in a fasting state is due to decreased gastric leptin level.

(1) Kojima *et al.* (1999) *Nature*, **402**, 656–660.

(2) Lee *et al.* (2002) *Endocrinology*, **143**, 185–90.

(3) Sakata *et al.* (2006) *J. Endocrinol.*, **190**, 749–57.

(4) Shimada *et al.* (2003) *Biochem. Biophys. Res. Commun.*, **302**, 520–525.

(5) Kamegai *et al.* (2004) *Regulatory Peptides*, **119**, 77–81.

(6) Toshinai *et al.* (2001) *Biochem. Biophys. Res. Commun.*, **281**, 1220–1225.

P11: Ghrelin; Leptin; Fasting; mRNA

TRANSCRIPTION FACTORS PROP-1, LHX2 AND LHX3 ACTIVATE FSH β GENE EXPRESSION

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Recently, we reported that a transcription factor Prop-1 stimulates the promoter activity of the porcine FSH β and α GSU genes (1,2). In addition, we cloned Lhx2 as another transcription factor of the FSH β gene and analyzed its function. In this meeting, we report the comparison of binding sequences and transcriptional activity of Prop-1, Lhx2 and Lhx3.

To determine binding sites and sequences of Prop-1, Lhx2 and Lhx3 in the FSH β promoter, electrophoretic mobility shift assay (EMSA) and DNase I footprinting analysis using fluorescence-labeled FSH β upstream fragments were carried out with recombinant protein of Prop-1, Lhx2 and Lhx3. Transfection assay in L β T2 cells was carried out using reporter vectors fused with several truncated FSH β upstream regions (-2320/+10 bp) to pSEAP2-Basic and expression vectors of Prop-1, Lhx2 and Lhx3 in pcDNA3.1.

EMSA and DNaseI footprinting analysis showed that Prop-1 and Lhx2 bind not only Fd2 region (-852/-746 bp) but also several regions of porcine FSH β promoter, which corresponded mostly to the regions as reported for Lhx3. Extensive comparison of the results represented that Prop-1, Lhx2 and Lhx3 share the same binding regions differing in the binding span and DNase I sensitivity. In addition, unique binding sites each for Prop-1, Lhx2 and Lhx3 were found. Transfection assay in L β T2 cells showed that Prop-1 and Lhx2 enhance the porcine FSH β promoter activity as well as Lhx3.

The present study demonstrated that Prop-1, Lhx2 and Lhx3 bind common and unique sequences in the FSH β promoter and stimulate its transcription. In consequence, the FSH β gene is likely to be modulated with several homeodomain transcription factors by sharing the common and unique regulatory regions.

(1) Aikawa *et al.* (2004) *Biochem. Biophys. Res. Commun.*, **324**, 946-952

(2) Sato *et al.* (2006) *J.Mol.Endocrinol.*, **37**, 341-352

P12: Prop-1, Lhx2, Lhx3, FSH β , Pituitary

IN VITRO SELECTION OF PITUITARY TRANSCRIPTION FACTOR PROP-1 RESPONSIVE ELEMENTS

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Transcription factor Prop-1 plays an essential role in the expansion of the pituitary primordium and the differentiation and development of the hormone-producing cells (1). Recently, we reported that Prop-1 binds to the upstream region of the porcine FSH β and α GSU genes and stimulates their promoter activities (2, 3). Notably, the upstream regions of porcine FSH β nor α GSU genes do not contain a consensus sequence for Prop-1 binding, PRDQ9 (5'-TAATgaATTA-3'). The present study aimed to reinvestigate the DNA binding specificity of Prop-1 by employing the SELEX (Systematic Evolution of Ligands by EXponential enrichment) using random N15 oligomers. Binding ability to Prop-1 was confirmed by electrophoretic mobility shift assay (EMSA) and *cis*-acting activity was evaluated by transient transfection assay using GH3 cells.

We found that Prop-1 binds to not only PRDQ9 but also various AT-rich sequences composing of AAAT-, AAAC-, TTAT-, TAAA-, TAAC-, and CAAT-N3-ATTA. EMSA and transfection assay revealed that Prop-1 bound to the identified sequences as a dimer and activated the transcriptional level. On the other hands, Prop-1 also binds to a sequence containing a single ATTA motif as a monomer but cannot enhance promoter activity.

The present results indicate that Prop-1 participates in the regulation of genes by binding to various AT-rich sequences containing ATTA motif, a consensus motif for homeodomain transcription factor, supplying with a couple of binding site.

(1) Sornson. *et al.* (1996) *Nature*, **384**,327-333

(2) Aikawa *et al.* (2004) *Biochem. Biophys. Res. Commun.*, **324**,946-952

(3) Sato *et al.* (2006) *J. Mol. Endocrinol.*, **37**, 341-352

P13: SELEX, Prop-1, Pituitary, PRDQ9, Homeodomain

DISTINCTION BETWEEN LIM HOMEODOMAIN TRANSCRIPTION FACTORS LHX2 AND LHX3

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Recently, we cloned a LIM homeodomain transcription factor Lhx2 as a *trans*-acting factor for the porcine follicle-stimulating hormone β subunit (FSH β) gene. As Lhx2 has been to regulate the pituitary glycoprotein hormone α subunit (α GSU) gene expression (1), our finding represents that Lhx2 regulates the FSH production at a transcriptional level. On the other hand, it is acknowledged that Lhx3 has similar effects on α GSU and FSH β genes (2, 3). In this study, we attempt to determine the Lhx2 and Lhx3 binding sequence in the α GSU and FSH β promoters, and to evaluate the *cis*-acting activity by transient transfection assay using L β T2 cells (gonadotrope cell line) and CHO cells (Chinese hamster ovary cell line).

DNase I footprinting analysis revealed that the binding sites of both factors are somewhat different. For example, in the α GSU promoter, Lhx2 and Lhx3 bound to -340/-329 and -340/-326 bp, respectively. The sequence AGCTAATTAA (-340/-329 bp) was common to them and contained TAAT and ATTA, a core motif of homeodomain transcription factors. DNase I footprinting analysis of both factors for FSH β promoter gave similar but subtle different profiles. Transient transfection assay showed that Lhx2 and Lhx3 activate α GSU and FSH β promoters in CHO cells. Notably, in L β T2 cells, Lhx2 represses α GSU promoter, while Lhx2 activates FSH β promoter and Lhx3 activated both promoters.

In summary, LIM homeodomain transcription factors Lhx2 and Lhx3 share the target sites in the same regions with differences in the binding mode and the effectiveness of gene expression.

(1) Roberson *et al.* (1994) *Mol. Cell. Biol.*, **14**, 2985-2993.

(2) Bach *et al.* (1997) *Genes. Dev.*, **11**, 1370-1380.

(3) West *et al.* (2004) *Endocrinology*, **145**, 4866-4879.

P14: Lhx2, FSH, Pituitary, Pig, Homeodomain

A NOVEL PITUITARY TRANSCRIPTION FACTOR, PAIRED-RELATED HOMEBOX FACTOR 2, REGULATES GONADOTROPIN SUBUNIT GENES

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We are currently attempting the cloning of transcription factors for porcine follicle-stimulating hormone β subunit (pFSH β) gene by the Yeast One-Hybrid System with a porcine anterior pituitary cDNA library using the Fd2 region (1) as a bait sequence. We previously cloned porcine (p) Prop-1 (2) and pLhx2, and recently succeeded in cloning of a porcine *paired*-related homeobox factor 2, pPrx2, as a transcription factor for pFSH β gene.

Our recent studies revealed that pProp-1 regulates porcine glycoprotein hormone α subunit (p α GSU) gene, but not porcine luteinizing hormone β subunit (pLH β) gene (3). This study focused whether pPrx2 regulates the gene expression of p α GSU and/or pLH β in addition to that of pFSH β .

Transient transfection assay using -1059/+12 bp of p α GSU gene, -2320/+10 bp of pFSH β gene and -1284/+7 bp of pLH β gene fused to pSEAP2-Basic showed an activation of promoter activities by pPrx2. Electrophoretic mobility shift assay and DNase I footprinting analysis using recombinant pPrx2 and FAM-labeled DNA fragments of p α GSU, pFSH β and pLH β promoters revealed that pPrx2 binds to all gonadotropin subunit promoters by recognizing the AT-rich sequences. Following transient transfection assay using a series of deletion mutants of three promoter regions in CHO cells demonstrated that some of the binding sites are responsive to pPrx2.

In summary, we succeeded in cloning of pPrx2 as a transcription factor which regulates FSH and LH at a transcriptional level. It is expected that pPrx2 provides novel clues to understand the mechanisms of gonadotropin subunit gene regulation.

(1) Kato *et al.* (1999) *Mol. Cell. Endocrinol.*, **158**, 69-78.

(2) Aikawa *et al.* (2004) *Biochem. Biophys. Res. Commun.*, **324**, 946-952.

(3) Sato *et al.* (2006) *J. Mol. Endocrinol.*, **37**, 341-352.

P15: Prx2, Gonadotropin, Pig, Pituitary, Homeobox

EXPRESSION AND CHROMATIN STRUCTURE OF GRANULOSA-CELL SPECIFIC GENES IN THE MOUSE OVARY

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The aim of this research is to investigate the mechanisms of gene expression in the reproductive organs. As a first step, we set to investigate the mechanisms of specific gene expression in reproductive tissues. We based our studies on the hypothesis that genes which are expressed in the same reproductive tissue present, at least to some extent, similar activation mechanisms.

This time, we focused our studies on the granulosa cells in the ovary. We cloned the *Amhr2*, *inhibin β B*, and *Scd2* genes and their expression patterns showed that they are highly expressed in the reproductive organs. Moreover, the genes *Amhr2* and *Scd2* showed to be expressed specifically in the granulosa cells in the ovary. Since recent studies have shown that the regulation of chromatin structure plays an important role in gene expression, we investigated the chromatin structure of these three genes. Investigation on the granulosa cells detected DNase I HS sites on the promoter region of *Inhibin β B* gene and on the intron 6 of *Amhr2* gene.

The comparison of the mouse *Amhr2* gene sequence to the human and the rat sequences showed high homology between DNase I HS sequence on the mouse *Amhr2* gene and the correspondent sequences on the human and rat genes. We found that the HS sequences in the *Amhr2* include binding sites to the transcription factors Sp1, NF-1, YY1, ISGF-3, NF-ATc, NF-ATp, NF-ATx, HNF-3alpha, RXR-alpha, VDR, ER-alpha, ER-beta, LF-1, and LXR-alpha that are also present in the *Inhibin β B* promoter. The current results suggest that these transcription factors may play an important role in gene regulation and in the activation of genes in granulosa cells.

**EXPRESSED SEQUENCE TAGS FROM THE OVARY OF THE RED SEA URCHIN
(*Pseudocentrotus depressus*)**

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The molecular nature of the gonad-stimulating substance (GSS) of the starfish has recently been clarified, and the goal of our study is to identify the GSS of the sea urchin, which is expected to be involved in oocyte growth and maturation. The sensitive methods for examining the effects of GSS candidates on oogenesis are essential to find the sea urchin GSS, and the microarray method for monitoring the expression levels of genes in the ovary is useful for this purpose. Here, cDNAs for spotting on microarray slides were isolated from the ovary of the red sea urchin (*Pseudocentrotus depressus*) and characterized.

Total RNA prepared from the ovary of the red sea urchin at the stage of mid oogenesis was reversetranscribed to cDNA. The redundancy of cDNA formed by abundant mRNA was reduced (normalization) by the Trimmer-Direct cDNA Normalization Kit (Evrogen JCS), and then cDNAs were amplified by PCR based on the SMART technique (Clontech laboratories. Inc.). A directional cDNA library was constructed, from which 960 clones were randomly one-pass sequenced from the 5'-end of the cDNAs. The average length of nucleotide sequences (ESTs) obtained was 644 bases, and 941 ESTs contained more than 100 bases. The 941 ESTs, assembled to create contigs from overlapping ESTs with the use of the genetyx-mac ATSQ program, formed 60 contigs from 132 ESTs; the remaining 809 ESTs were singletons, and the contigs were made from 4 ESTs at most. Thus, 869 independent sequences were obtained, which strongly suggesting that the library was successfully normalized. The public database of DNA sequences was searched by the blast programs to find counterparts of ESTs. About 70 % of ESTs showed significant homologies (E-value, less than 1E-20) to deposited sequences. The highest matches were seen mostly against sequences from the purple sea urchin (*Strongylocentrotus purpuratus*) whose whole genome sequence has been completed. The variety of cDNAs obtained in this study could be of great use in the establishment of a microarray method for evaluating the gene expression profiles during oogenesis in the red sea urchin.

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P17: EST, sea urchin, ovary, oogenesis

SURVEY OF PITUITARY HORMONES ON THE HATSCHEK'S PIT OF AMPHIOXUS

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Amphioxus, which is categorized into subphylum Cephalochordata, is known as the phylogenetically closest invertebrate to vertebrates. Hatschek's pit, which exists in a right side of notochord in the amphioxus head, is considered as the putative homologue of the pituitary of vertebrates because of the presences of secretory granules and immunoreactive cells against a human luteinizing hormone. Furthermore, the role of Hatschek's pit has been suspected to be important in the reproductive functions, which might be occurred by thermal, chemical, or pheromonal cues, based on its structure opening to the water currents in the mouth cavity(1). However, direct evidence on the pituitary function has never been demonstrated. In order to study on the function of the Hatschek's pit, we attempted to survey the tissue specific genes expressed in the pit of amphioxus *Branchiostoma belcheri*.

Hatchek's pit is very small organ which is 300µm in length, and strongly connected with epithelial tissues. It is difficult to anatomically remove the only pit by using ordinary dissection methods. We used a novel technology of Laser-microdissection (LMD), which is able to dissect selected tissues, to collect the only pit. One hundred forty pieces of cross sections of frozen amphioxus heads were prepared on a few slide glasses from fifteen animals. Whole tissues of Hatchek's pit were cut by using LMD and collected in a tube. Total RNA was extracted from the collected sample and we constructed cDNA library by a method of subtractive-hybridization, which using suppression PCR method. By sequencing two hundreds of subtracted clones, five clones which belonged in different gene families were obtained as candidates for genes specifically expressed in the pit. These clones showed homologies to cDNAs of amphioxus *Branchiostoma floridae* which are placed in an EST database. However, the specific expression of these clones did not demonstrate only in Hatchek's pit. We continue to survey the specific genes on the pit by sequencing more number of remaining clones, and try a differential cloning method.

(1) Gorbman, A. *et al.* (1999) *Gen. Comp. Endocrinol.*, **113**, 251-254.

P18: amphioxus, Hatschek's pit, Laser-microdissection

MOLECULAR IDENTIFICATIONS OF THREE ISOFORMS OF PPARS (PEROXISOME PROLIFERATOR-ACTIVATED RECEPTORS) IN THE LEOPARD GECKO, *Eublepharis macularius*.

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In gekkonid lizards, a tail is an important site for lipid storage and is indispensable for their survival in desert environments (1). In order to reveal the molecular mechanism of lipid metabolism in tails of the lizards, we focused on peroxisome proliferator-activated receptors (PPARs). PPARs are the members of superfamily of the nuclear hormone receptors. A great number of studies in rodent and human have shown that PPARs were involved in the lipids metabolism.

In this study, we identified a sequence of leopard gecko (lg) PPAR γ cDNA and partial sequences of PPAR α and PPAR β cDNA containing a DNA binding domain. In addition, we identified a sequence of PPAR γ 2 variant. The amino acid sequence deduced from lgPPAR γ cDNA shows a high similarity with its corresponding peptides of crocodile, turtle, mammals, and birds. In the phylogenetic tree drawn with this sequence and its corresponding sequences of mammals, birds, reptiles and fishes, the leopard gecko clustered with reptiles and birds. The RT-PCR analysis revealed that all three isoforms of PPARs was detected in all tissues examined, except that PPAR γ 2 was found only in the abdominal fat body and tail adipose tissue. The competitive PCR assay revealed that lgPPAR γ mRNA was highly expressed in the abdominal fat body, tail adipose tissue and tail muscle, whereas lgPPAR γ 2 shows less expression in the adipose tissue in comparison with total expression of lgPPAR γ . lgPPAR α showed high expression in the liver and lgPPAR β showed high expression in the brain and liver. These results may reflect the difference of their physiological functions. PPAR γ is mainly associated with lipid storage in adipose tissues, while PPAR α and PPAR β are associated with fatty acid oxidation.

(1) Bustard. H.R. (1967) *Science*, **158**, 1197-1198.

P19: PPAR、Lipid、Metabolism、Reptile、Tail

MASS ANALYSIS OF SECRETORY PROTEINS EXPRESSED IN THE EARLY FORMATION OF THE PITUITARY OF FETAL RATS

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The hypothalamic-pituitary axis plays a pivotal role in vertebrate endocrine systems by integrating signals from the environment and the brain. The pituitary gland is composed of an adenohypophysis (the pars distalis, pars intermedia, and pars tuberalis) and a neurohypophysis (the pars nervosa). The former is derived from an invagination of the oral ectoderm of the stomodeum, known as Rathke's pouch, whereas the latter is an infundibulum of the diencephalon. Hormones in the pars distalis (PD) are regulated by hypothalamic neurohormones that are produced in the hypothalamic area and transported to the PD via portal vessels. The PD is vascularized by hypophysial portal vessels that arise from the capillary beds in the median eminence of the hypothalamus, and this hypophyseal portal system provides an important link for carrying hormonal information from the central nervous system to the pituitary. The capillaries of the PD are characterized by richly fenestrated endothelia.

The results of a previous study by our group suggest that vascular endothelial growth factor (VEGF-A), which is known to have a mitogenic effect or guidance effect on the endothelial cells, is involved in the development of the primary capillaries and in the vascularization of the PD, but not in the development of the portal vessels between hypothalamus and pituitary. This latter conclusion is based on the observation that the formation of portal vessels begins at E13.5, before the expression of VEGF-A in the rostral region of the pars distalis (1). The assumption drawn, therefore, was that other signal molecules may be involved in the formation of the pituitary portal vessels. In the present study, we extensively analyzed secretory proteins by the Signal Sequence Trap method using rat pituitary anlage at E13.5, the point at which the formation of the portal vessels starts. We cloned and characterized about 500 clones, eventually identifying several unknown molecules. Spatiotemporal expression of unknown molecules was examined by *in situ* hybridization and RNA dot blot analysis. We expect that novel growth factors other than VEGF-A will be identified in near future.

(1) Nakakura *et al.* (2006) *Cell Tissue Res.*, **324**, 87-95.

Key words: pituitary gland, hypophyseal portal system, formation of portal vessels, VEGF-A, Signal Sequence Trap method,

SELECTIVE COLLECTION OF CATECHOLAMINERGIC (CA) NEURONS IN THE BRAIN AND ITS APPLICATION TO GENE EXPRESSION ANALYSES

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In the brain, catecholaminergic (CA) neurons are localized in discrete regions including the olfactory bulb (OB), the ventral tegmental area (VTA), the substantia nigra (SN), the arcuate nucleus of the hypothalamus, the locus coeruleus (LC), and the nucleus of the solitary tract (NTS). Among these neurons, those in the brain stem and/or the hypothalamus are implicated in physiological functions including feeding, sleep and arousal, emotional actions, and reproductive functions, as well as stress responses. We have been studying the functional roles of the CA neurons in these regions using animal models. In the present study, the tyrosine hydroxylase (TH)-green fluorescence protein (GFP) transgenic mouse (TH-GFP mouse) was used in order to characterize the gene expression profile of CA neurons in the brain stem. The GFP-labeled NA neurons in the fetal LC were collected with high precision, i.e., 95% or more, both in specificity and selectivity, using flow cytometry (FACS). We made comprehensive analysis of transcripts expressed in the NA neurons in the LC using GeneChipTM (Affymetrix), and identified transcripts that are specifically expressed in the LC. The result of the present study provides us with a new method for analyzing extensively the gene expression profiles in this single species of NA neurons in a highly specific manner.

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Key wards: Micro array, flow cytometry, catecholamines, green fluorescence protein

COMPARATIVE ANALYSIS OF THE GONADAL AND PITUITARY GnRH SYSTEMS IN CHORDATES

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GnRH was originally identified as a hypothalamic decapeptide, gonadotropin-releasing hormone, which stimulates pituitary gonadotropes to synthesize and release gonadotropins. GnRH and its receptor are expressed in various extrapituitary sites, suggesting that GnRH has diverse physiological functions. We identified genes encoding GnRH ligands and receptors from various animal classes of chordates. The results revealed that gene numbers of both ligands and receptors remarkably differ among and in some cases within animal classes. This indicated that comparative approaches are indispensable to clarify the complex physiology of GnRH systems so as to understand generality and species specificity of physiological functions of GnRH. In these situations, we found that despite differences in gene numbers, the expression of GnRH receptor in gonads is well-conserved in many chordate classes including Ascidiacea. It was hypothesized that physiological roles of GnRH in gonads would be an early evolved function originating before the emergence of the pituitary gland and have been conserved significantly. In this study, we characterized GnRH systems in the anterior pituitary gland and gonads in chordates by focusing on the leopard gecko *Eublepharis macularius*, a species with two GnRH isoforms and three GnRH receptor subtypes. The quantitative RT-PCR analysis revealed that only one receptor subtype was expressed in the anterior pituitary gland. Meanwhile, all the three receptor subtypes were expressed in gonads with high levels. Seasonal and developmental changes in the expression of GnRH receptors were observed in ovarian follicles. *In situ* hybridization showed spatial expression patterns of ovarian receptors and suggested co-expression of multiple receptor subtypes in granulosa cells. Co-transfections of multiple receptor subtypes showed distinct pharmacologies in COS-7 cells compared with those of single transfections, possibly due to interaction and signaling crosstalk between multiple receptor subtypes. These findings combined with results in other chordate species suggest that distinct signaling mechanisms are involved in the gonadal and pituitary GnRH systems. Molecular diversity of GnRH systems and combination of GnRH ligands and receptors may largely contribute to the functional diversity of GnRH.

P22: GnRH, leopard gecko, reptile, pituitary gland, evolution

CLONING AND CHARACTERIZATION OF cDNAs ENCODING PITUITARY ADENYLATE CYCLASE-ACTIVATING POLYPEPTIDE FROM THE TELEOST AND ELASMOBRANCH FISH

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Pituitary adenylate cyclase-activating polypeptide (PACAP) is widely distributed in the central nervous system (CNS) and peripheral tissues, and is involved in many physiological processes, such as cell proliferation, differentiation, cell death, neurotransmission and neuroprotection in mammals (1-3). On the other hand, PACAP plays a role in mediating pituitary hormone release in the teleost fish (4). The aim of the present study was thus to use the teleost and elasmobranch fish, fish models employed to study the neuronal actions of PACAP, to examine the structural characterization of PACAP in fish. We examined the nature of PACAP cDNAs isolated from the goldfish, Japanese dace, stargazer and stingray. The primary structure of PACAP deduced from its cDNA is highly conserved among these species, while that of growth hormone releasing hormone which is also encoded in PACAP cDNA has a few variation. We also observed the distribution of PACAP mRNA in fish. PACAP mRNA expression was mainly found in the CNS and visceral organs such as the heart, stomach, intestine and gonads.

- (1) Arimura (1998) *Jpn. J. Physiol.*, **48**, 301-331.
- (2) Sherwood *et al.* (2000) *Endocr. Rev.*, **21**, 619-670.
- (3) Vaudry *et al.* (2000) *Pharmacol. Rev.*, **52**, 269-324.
- (4) Montero *et al.* (2000). *J. Mol. Endocrinol.*, **25**, 157-168.

Keyword: PACAP, GHRH, cDNA, teleost, elasmobranch

NUCLEOTIDE SEQUENCES OF PROOPIOMELANOCORTIN SUBTYPE GENES IN BARFIN FLOUNDER, PLEURONECTIFORMES

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Proopiomelanocortin (POMC) is a precursor of melanocortins (MCs) such as melanophore-stimulating hormone (MSH) and adrenocorticotrophic hormone (ACTH), which contains an essential sequence, His-Phe-Arg-Trp, and a single β -endorphin (β -END).

POMC is mainly produced in the corticotropes of pars distalis and the melanotropes of pars intermedia of the pituitary, in which tissue-specific cleavage and modification take place; ACTH and β -END are preferentially generated in the pars distalis and MSHs and N-terminally acetylated β -END in the pars intermedia (1). One transcription factor, the Tbox factor Tpit, is critical for the differentiation of two POMC lineages and expression of the POMC gene (2). Recently, we found that the barfin flounder, *Pleuronectiformes*, expresses three subtypes of POMC (A, B, and C) in the pituitary (3). The expression of POMC-A was restricted in the pituitary, while POMC-B and -C were expressed in the brain and many peripheral tissues. The present study was undertaken to determine the gene structure of barfin flounder POMCs as a first step to examine functional differentiation of the POMCs.

The 5'-flanking regions of POMC subtype genes were amplified from genomic DNA prepared from barfin flounder liver by cassette polymerase chain reaction (PCR). POMC-A (-648 to -1) contained one TATA box, two cAMP response element-like elements (CRE-like), two Eboxes and one retinoic acid-inducible element. POMC-B (-958 to -1) contained one TATA box, one CCAAT box, four E boxes, one CRE-like, and one binding site for Tpit. POMC-C (-584 to -1) contained one TATA box, two CCAAT boxes, two E boxes, and one binding site for Tpit. There were no similarities in the distribution patterns of these *cis*-elements among the three POMC genes. Nucleotide sequences of introns were determined using DNA amplified from genomic DNA by PCR. POMC-A, B, and C genes were composed of three exons and two introns. In these genes, an open reading frame was encoded in the second and third exons, and all MSH and β -END segments were encoded on the third exon. These structural characteristics are similar to POMC genes of other vertebrates (1). In the barfin flounder, intron B of POMC-A and -B genes contained microsatellites consisting of a CA repeat. The molecular architecture of the POMC gene may have been established in early vertebrates, and present vertebrates have inherited it. In the barfin flounder strain, at least two duplications may have occurred on the POMC gene; ancestral POMC-A/B and POMC-C occurred at the first duplication, and POMC-A/B split into POMC-A and POMC-B at the second duplication. The CA repeat may have been inserted into intron B of ancestral POMC-A/B. The difference in tissue distribution of POMC genes may result from the diversification of *cis*-elements in the 5'-flanking region.

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- (2) Drouin. (2006) In: A. Arzt *et al.* (eds.), *Pituitary Today*, vol. 35, pp. 74-87.
- (3) Takahashi *et al.* (2005) *Gen. Comp. Endocrinol.*, **141**, 291-303.

Key word: proopiomelanocortin, intron, exon, *cis*-elements, barfin flounder.

RELATIONSHIP BETWEEN ANAL RESPIRATION AND NEUROSUBSTANCES IN THE CRAYFISH, *Procambarus clarkii*

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“Anal respiration” in the crayfish has been described in the book "The Crayfish: An Introduction to the Study of Zoology" published in 1880. The observation has been noted that water is alternately taken into and expelled from the anus fifteen to seventeen times in a minute and it seemed to be regulated by the sixth abdominal ganglion.

To investigate neurosubstances included in the sixth abdominal ganglion, topological mass spectrometry analysis was available. Direct matrix-assisted laser desorption/ionization with time-of-flight mass spectrometry with slice of the sixth abdominal ganglion was resulted in the presence of orcokinin and its gene-related peptides, crustacean-SIFamide, decapod tachykinin-related peptide, and crustacean cardioactive peptide. These peptides, together with acetylcholine, γ -amino butyric acid and L-glutamic acid, were examined *in vivo* on bioassays for rhythmic anal contraction and anal water flux. The activity of the peptides on the isolated intestine was also examined *in vitro*. Rhythmic anal contraction was caused by orcokinins, decapod tachykinin-related peptide, and acetylcholine, when each sample of 100 pmol was injected into the crayfish. Crustacean cardioactive peptide had most potent activity of anal opening that was kept for fifteen seconds or more. Water flux through the anus was observed by injection of crustacean cardioactive peptide or decapod tachykinin-related peptide. Interestingly, the action of the neurosubstances on the isolated intestine was diversified. The contraction of hindgut was activated by orkokinins and acetylcholine, but not by crustacean cardioactive peptide. Decapod tachykinin-related peptide showed repetitive action alternating between contraction and relaxation on the rectum. Crustacean-SIFamide caused complex movements of the hindgut, and induced most potent activity of defecation.

The crayfish can take water from the anus, but each function of neurosubstances identified in sixth abdominal ganglion remains unclear. We think that “anal respiration” might not be a normal phenomenon, since the action which is alternately taken into and expelled from the anus fifteen to seventeen times in a minute was able to be reproduced by the overdose of a combination with crustacean-SIFamide and decapod tachykinin-related peptide.

P25: crayfish, anal respiration, neuropeptide

PURIFICATION, KINETIC CHARACTERIZATION, AND MOLECULAR CLONING OF A NOVEL ENZYME ECDYSTEROID 22-KINASE

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It has been suggested that the ovaries of most insect species have the capacity to accumulate ecdysteroid phosphates, which are physiologically inactive, and the ecdysteroid phosphates function as a source of free active ecdysteroid before the prothoracic glands differentiate during embryonic development (1). Recently, in the silkworm *B. mori* eggs, a novel enzyme ecdysteroid-phosphate phosphatase (EPPase), which is specifically involved in dephosphorylation of ecdysteroid phosphates, was isolated, characterized and revealed to be a vital enzyme that may control the “on/off switch” of embryonic development (2). In this study, ecdysteroid 22-kinase (EcKinase) was purified from the cytosol of the *B. mori* ovaries, and the biochemically characterized. Results obtained indicated that the reciprocal conversion of free ecdysteroids and ecdysteroid 22-phosphates by two enzymes, EcKinase and EPPase, plays an important role in ecdysteroid economy of the ovary-egg system of *B. mori*. On the basis of the partial amino acid sequence obtained from purified EcKinase, the nucleotide sequence of the cDNA encoding EcKinase was determined. The full-length cDNA of EcKinase was composed of 1,850 bp with an open reading frame encoding a protein of 386 amino acid residues. A database search showed that EcKinase has an amino acid sequence characteristic of phosphotransferases (Brenner's motif), but there are no functional proteins that share high identity with the amino acid sequence of EcKinase (3).

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(3) Sonobe H. *et al.* (2006) *J. Biol. Chem.*, 281, 29513-29524.

P26: Molting hormone, Ecdysteroid, Ecdysteroid 22-kinase, Phosphorylation of steroid hormone, Silkworm

PRIMARY STRUCTURE AND POST-TRANSLATIONAL PROCESSING OF PROOPIOMELANOCORTIN IN DOGFISH, *TRIAKIS SCYLLIUM*

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In tetrapods, proopiomelanocortin (POMC) is composed of adrenocorticotropin (ACTH), β -lipotropin (β -LPH) and *N*-POMC segments. Each segment contains one MSH; α -MSH in ACTH, β -MSH in β -LPH, and γ -MSH in *N*-POMC. β -LPH also contains β -endorphin (β -END). The pituitary is a major tissue producing POMC; however, POMC-derived peptides of the pars distalis (PD) differ from those of the pars distalis (PI). Final products in the PD are *N*-POMC, ACTH and β -END, and those in the PI are MSHs and N-terminally acetylated and C-terminally truncated β -END. In cartilaginous fish, the primary structure of POMC has been shown in Holocephalans (Chimaeriformes) (1) and Elasmobranchs (Heterodontiformes, Squaliformes, and Rajiformes) (2-4). They characteristically have an additional MSH (termed δ -MSH) in the β -LPH segment; however, no information is available for the post-translational processing of POMC. In the present study, we first determined the primary structure of POMC by cDNA cloning in dogfish, *Triakis scyllium*, of Lamniformes, one of the five orders of Elasmobranchii, and then screened POMC-derived peptide in PD and PI with MALDI-TOF mass spectrometry to evaluate post-translational processing of POMC in cartilaginous fish.

Triakis pituitary cDNA library was prepared using the pGCAP10 vector. POMC cDNA was identified with BLAST analysis of 1536 clones. *Triakis* prePOMC contained four MSHs and a single β -END as in other cartilaginous fish POMCs and showed the highest sequence identity (72%) with *Squalus* and *Heterodontus* prePOMC. All hormonal segments flanked by basic amino acids also showed the highest sequence identity with *Squalus* peptides excluding β -MSH, which was most similar to the *Heterodontus* peptide. MALDI-TOF mass spectrometry was performed using extracts of rostral PD (RPD) and PI. As a result, ACTH, β -END, and joining peptide (JP) were detected in the extract of the RPD, and α -MSH, β -MSH, β -MSH₁₋₁₆, γ -MSH, δ -MSH, β -END, β -END₁₋₃₀, JP, and some other POMC-derived peptides were identified in the extract of PI; however, neither acetylated α -MSH or acetylated β -END was detected in PI.

In summary, *Triakis* POMC has the same molecular architecture as other cartilaginous fish. Post-translational processing of *Triakis* RPD differs from that of PI as shown by the identification of differential final products. These tissue-specific processing mechanisms are similar to other vertebrate pituitaries. No detection of acetylated peptides suggests the lack of an acetylation system in melanotrophs of the *Triakis* pituitary.

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(2) Amemiya *et al.* (2000) *Gen. Comp. Endocrinol.*, **118**, 105-112.

(3) Dores *et al.* (2003) *Gen. Comp. Endocrinol.*, **133**, 71-79.

(4) Takahashi *et al.* (2004) *Gen. Comp. Endocrinol.*, **138**, 159-165.

P27: proopiomelanocortin, post-translational processing, cartilaginous fish, *Triakis*.

OPERATION OF THE TISSUE-TYPE PLASMINOGEN ACTIVATOR /PLASMIN SYSTEM IN THE MEDAKA OVARY

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It is well known that tissue-type plasminogen activator /plasmin (tPA/Pln) system participates in fibrinolysis. Plasminogen is mainly expressed in the liver and secreted into the blood stream. Plasminogen is converted to plasmin by tPA or urokinase-type plasminogen activator (uPA). Previous reports have demonstrated the existence of the tPA/Pln system in reproductive organs in mammals. However, their physiological functions in the reproduction remain to be elucidated.

In this study, to understand the physiological roles of the tPA/Pln system in reproduction, we used medaka ovary. We first cloned medaka plasminogen cDNA and generated its specific antibody using a recombinant protein. Western blot analysis showed that plasminogen protein is constantly expressed in the medaka ovary during the daily ovulatory cycle except for immediately before ovulation. In contrast, the activity of plasmin, the mature enzyme form of plasminogen, was detected at the 5 and 9 hours after ovulation by gelatin zymographic analysis. This suggests that the medaka plasmin is involved in follicular degradation after ovulation. Because mammalian plasmin effectively degrades extracellular matrix proteins like fibronectin and laminin, we examined whether medaka plasmin degrades extracellular matrix in the follicle. The results showed that plasmin could degrade type I collagen, fibronectin, laminin, and fibrinogen. These data suggest that plasmin may participate in follicle degradation after ovulation.

P28: Plasminogen , tissue-type Plasminogen Activator, follicular degradation

ANALYSIS OF MEDAKA KALLIKREIN LIKE PROTEIN THE REPRODUCTIVE ORGANS

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Kallikreins are a subgroup of serine protease and these proteolytic enzymes have diverse physiological functions in many tissues. Growing evidence suggests that many kallikreins are potential biomarkers for many human diseases. Most of them are found to be under hormonal regulation. Studies on kallikreins have been extensively done on mammals such as human, mouse, and rat. Lower vertebrate species like medaka could serve as good experimental models for the study of unresolved problems in biology common to vertebrates.

Recently we cloned a novel serine protease from a medaka ovary cDNA library by screening followed by 5'-RACE. The primary amino acid sequence showed a 59% homology with a serine protease from brook trout and nearly 30% with mouse tissue kallikreins. Northern blot analysis revealed that the novel protease is heavily expressed in the ovary and testis of the adult medaka. RT-PCR analysis demonstrated that, in the ovary, the gene is expressed in all stages of follicular development and in ovulated follicles.

In situ hybridization analysis detected the transcript in theca cells of the ovarian follicles and in somatic cells of the testis.

We produced the recombinant protein of this enzyme in *E.coli* and raised a polyclonal antibody against the protein in rats. Western blot analysis done using the whole ovary extracts after ovulation could detect a study expression of the protein for a long period, and the protein level significantly decreased prior to the next ovulation. Subsequent immunohistochemical analysis detected the protein in the theca cells of the ovulated follicles. These results suggest that this protease may have a function in the theca cells of the follicle that has released the ovum.

P29: medaka, serine protease, kallikrein, ovulation.

PROCESSING OF GONADOTROPIN-INHIBITORY HORMONE IN THE QUAIL HYPOTHALAMUS

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Recently, we identified a novel hypothalamic neuropeptide with a C-terminal LPLRFamide sequence in the quail brain (1). This avian neuropeptide was shown to inhibit gonadotropin release from the cultured anterior pituitary. This peptide is the first hypothalamic peptide that inhibited gonadotropin release reported in vertebrates. We, therefore, termed it gonadotropin-inhibitory hormone (GnIH). After this finding, we found that GnIH-related peptides were present in the brains of other vertebrates, such as mammals, birds, amphibians, and fish (2-8). These GnIH-related peptides possessed a LPXRFamide (X=L or Q) motif at their C-termini in all investigated animals.

We further characterized the GnIH cDNA from the quail brain and found that the deduced GnIH precursor polypeptide encoded one GnIH and two gene-related peptides (GnIH-RP-1 and GnIH-RP-2). These peptides possessed a LPXRFamide (X=L or Q) motif at their C-termini. Subsequently, GnIH-RP-1 and GnIH-RP-2 were identified as mature endogenous peptides using mass spectrometric analyses. In addition, N-terminally extended forms of GnIH and GnIH-RP-2 were found to be present in the quail hypothalamus.

In the present study we found that GnIH and GnIH-RP-2 were processed from their N-terminally extended forms in the hypothalamus by biochemical and immunohistochemical analyses. This result suggests that the unique N-terminal processing mechanism may be present in the precursor polypeptide of GnIH in the quail hypothalamus.

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(5) Ukena *et al.* (2003) *Endocrinology*, **144**, 3879-3884.

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P30: Brain, Hypothalamus, Neuropeptide, Avian, GnIH

DETAILED ANALYSIS OF LOCALIZATION OF ACYLATED AND DES-ACYLATED GHRELIN IMMUNOREACTIVITY IN RAT GASTRIC GHRELIN CELLS

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Ghrelin was isolated as an endogenous ligand for growth hormone secretagogue receptor (GHS-R) from the rat and human stomach (1) and it exists as two forms: acylated ghrelin (AG) and des-acylated ghrelin (DG) (2). Exogenous administration of fatty acid increased AG production in the stomach (3). However, the intracellular region of ghrelin modification is still unclear. To clarify this point, we studied the detailed localization of AG and DG in gastric ghrelin cells by immunofluorescence double staining.

AG and DG were distributed in a heterogeneous pattern in the cytoplasm of ghrelin cells. Strong immunochemical staining with AG was found in the region under the plasma membrane, whereas DG was localized throughout the cytoplasm with strong signals in the juxtannucleus region. Interestingly, AG and DG were colocalized in the intermediate region between the plasma membrane and nucleus. These results indicate that the two forms ghrelin localize in different intracellular regions and that modification of DG by fatty acid may occur during transport from the juxtannucleus region to the plasma membrane.

(1) Kojima, M. *et al.* (1999) *Nature*, **402**, 656-660.

(2) Hosoda, M. *et al.* (2000) *Biochem. Biophys. Res. Commun.*, **279**, 909-913.

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P31: acylated ghrelin, des-acylated ghrelin, immunofluorescence

IDENTIFICATION OF STEROIDOGENIC ENZYMES IN THE OVARY OF AMPHIOXUS

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Sex steroids play the essential roles on the reproductive phenomena of vertebrates. The synthetic pathway of sex steroids is commonly distributed in vertebrates, from agnate to mammals, but has not found in invertebrates. The origin of sex steroidogenesis in vertebrates is considered to be evolved from the reproductive system in ancestral animal of vertebrate such as protochordate. The urochordate is a candidate of the animal in which the sex steroids regulate the reproduction. However, in the genomic sequences of ascidian *Ciona intestinalis*, have not found any genes related to the sex steroid biosynthesis.

In the molecular phylogenetic analysis, cephalochordata is known to be the closest invertebrate relative to vertebrate, in comparison with urochordate. We examined the presence of sex steroidogenic pathway in amphioxus *Branchiostoma belcheri*, which belongs to the subphylum cephalochordata of the phylum chordate. We isolated the cDNAs encoding steroid metabolic enzymes such as cytochrome P450 side chain cleavage enzyme (CYP11A), cytochrome P450 17 α -hydroxylase/17, 20-lyase (CYP17) and cytochrome P450 aromatase (CYP19) from ovary. By the results of multiple alignment and molecular phylogenetic analysis, amphioxus CYP enzymes were considered to be the ancestral forms of those in vertebrates.

To confirm the presence of sex steroids metabolizing pathway, *in vitro* conversion of ¹⁴C-labeled precursor steroids was examined by the extracts of amphioxus ovaries. The conversions from pregnenolone (P5) to progesterone (P4), P4 to androstenedione (AD), and AD to testosterone (T), estrone (E1) and estradiol-17 β (E2) were clearly detected by using a thin layer chromatography. These results demonstrated that CYP17 and CYP19 which we cloned from amphioxus ovary act on sex steroid synthesis. Furthermore, 3 β -hydroxysteroid dehydrogenase (3 β -HSD) and 17 β -HSD which we have not cloned yet also present and act in amphioxus.

It is strongly suggested that serial sex steroid synthetic pathway from cholesterol to E2 via P5, P4, AD and T is present in the ovary of amphioxus as same as in vertebrates. This is the first finding of the vertebrate-like synthetic pathway of sex steroids in invertebrate species.

P32: amphioxus, ovary, sex steroid, steroidogenic enzyme

THE RARE OCCURRENCE OF A SPONTANEOUSLY MATURING EEL IN CAPTIVITY

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Unlike many fish, eels (*Anguilla* spp.) do not normally undergo gonadal development under culture conditions and gonadal maturation has therefore been induced artificially using injections of gonadotropin-like substances. We recently stumbled across an exception to this dogma as a Japanese eel, (*A. japonica*) which had been reared long-term in sea water, matured without exogenous hormone treatment. Here, we report on the fertility and appearance of the gonads from this spontaneously maturing eel.

Eight 3-year-old eels were purchased from a commercial company and reared in ambient sea water under natural light conditions for 2.5 years. One fish matured spontaneously, and its gonads consisted of a number of testicular lamella and a total of 7 lumps of gonadal tissue. The presence of spermatozoa in the testicular compartment was apparent. To investigate fertility, gonads were minced into small pieces with scissors and milt was collected. The milt was maintained in artificial seminal plasma (1) at 4 degrees, and used to determine motility and to fertilize ovulated eggs when obtained from artificially matured female eels. The survival rate of larvae 10 days after hatching was subsequently recorded.

Gonadosomatic index (GSI) of the spontaneously maturing eel was 18.4%, whereas that of the other 7 immature fish, all male, was less than 0.18%. Histologically, the gonads of the maturing eel had male germ cells in all stages of development, as well as a large number of early vitellogenic oocytes. Sperm motility was 87.1%, and gametes had fertilizing capability as reflected in the acquisition of hatched larvae. The larvae grew normally and some of them underwent metamorphosis into glass eel. Albeit rare, we have shown that a naturally maturing eel under cultivation conditions could produce milt that had fertilizing capability.

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P33: natural maturation, eel, spermatogenesis, oocyte

STRUCTURE OF MEDAKA COLLAGEN AND ITS EXPRESSION IN THE OVARY: COMPARISON WITH MAMMALIAN OVARY

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Ovulatory process is an essential prerequisite for fertilization and subsequent embryonic development. The climax of ovulation is the rupture of follicle wall, leading to the release of a fertilizable ovum. However, it has not been known over the past century what is the mechanism of follicle wall rupture. Previous studies on mammalian ovaries demonstrated that the follicle wall abundantly contains extracellular matrix (ECM) proteins. Therefore, degradation of those ECM embedded in the wall is thought to be essential for follicle rupture during ovulation. Recently, we have demonstrated that follicle rupture in the medaka involves the cooperation of at least three matrix metalloproteinases (MMPs) and the tissue inhibitor of metalloproteinase-2b protein. In this study, we have attempted to determine the distribution of type I collagen in the ovary of the fish and a correlation between type I collagen $\alpha 1$ degradation and follicle rupture during ovulation.

A cDNA clone for type I collagen $\alpha 1$ was isolated from a medaka ovary by RT-PCR using degenerate primers synthesized based on the available partial nucleotide sequence of medaka type I collagen $\alpha 1$ chain followed by 5' and 3'-RACE. The clone for medaka type I collagen $\alpha 1$ (5124bp) was found to code for a protein of 1446 amino acids. The primary sequence showed 80% homology with human type I collagen $\alpha 1$ and nearly 88% with *Paralichthys*. RT-PCR analysis conducted for various tissues demonstrated abundant expression of type I collagen $\alpha 1$ in ovary of the mature medaka. *In situ* hybridization analysis detected the transcript in the follicle layer. Type I collagen $\alpha 1$ was observed in the theca cells by immunohistochemistry. The requirement of degradation of type I collagen $\alpha 1$ during ovulatory process was examined by the analysis of the protein in follicle layers collected before and after ovulation. Western blot analysis using a specific antibody for type I collagen $\alpha 1$ indicated that the amount of type I collagen $\alpha 1$ protein was higher in follicle layers before ovulation compared to that of after ovulation. These observations are the first direct evidence that the degradation of type I collagen in the follicle layer of ovulating follicles is essential for ovulation.

P34: follicle rupture; ovulation; type I collagen degradation; medaka fish

GENE EXPRESSION PATTERN IN TILAPIA GONADS DURING SEX DIFFERENTIATION

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The molecular mechanism underlying gonadal sex differentiation has been little known throughout vertebrates. This study was aimed to demonstrate gene expression patterns which were involved in gonadal sex differentiation in XX and XY gonads during early developmental stages of the Nile tilapia. In this study, all genetic XX group and XY group were used, then gonadal ridges were isolated surgically throughout experimental period. Total RNA was extracted from each gonads, then used for one-step real time RT-PCR analyses. In the XX gonads, *foxl2* and aromatase (*cyp19a1*) were upregulated after 5 to 7 days post hatching (dph). All other steroidogenic enzymes, such as cholesterol side-chain cleavage, 3 β -hydroxysteroid dehydrogenase (3 β -HSD), steroid 17 α -hydroxylase / c17-20 lyase, 17 β -HSD type1, were gradually upregulated in both XX and XY gonads during this period. These results indicated that estrogen production began only in the XX gonads after 5 dph, and which was regulated by *cyp19a1* expression that was under-controlled by *foxl2* expression. The XY gonads also gradually increased an ability for producing androgen during this period as same as XX gonads did. However, after 10 dph, the expression levels of all steroidogenic enzymes in the XY gonads maintained relatively low until 35 dph, in contrast to the fact that XX gonads further increased expressions of all steroidogenic enzymes during this period. These results probably indicated that the steroidogenic activity was much more active in the XX gonad during 10 to 35 dph. In contrast to dimorphic expression pattern in steroidogenic enzymes between XX and XY gonads, expression levels of steroid receptors, such as estrogen receptor α , β 1 and β 2, and androgen receptor α and β did not show significant differences between the sexes. Taking all together, tilapia ovarian differentiation is regulated by estrogen production during early developmental stages from 5 to 35 dph, however XY gonads may not be necessary to produce androgen during this period for differentiating toward testis.

P35: Nile tilapia, gonad, steroidogenesis, sex differentiation, steroid receptor

HSP90 β IS INVOLVED IN SIGNALING PROLACTIN-INDUCED 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 just before the initiation of meiosis during newt spermatogenesis, but the underlying molecular mechanism remained unknown. The aim of this study is to determine the role of heat shock protein (HSP) 90 β in prolactin-induced apoptosis. HSP90 β is a molecular chaperone for client proteins in signal transduction and transcription to regulate cell proliferation and survival. Several lines of evidence indicate that HSP90 β protects cells from apoptosis. First, in order to explore if HSP90 β is expressed in newt testis, we cloned HSP90 cDNA, which is highly homologous with those of other species, from newt testis by RT-PCR. To reveal the relationship of HSP90 β and prolactin receptor in prolactin-induced apoptosis, we examined the expressions of their proteins in the spermatogenic stages and cell types of newt testis by Western blotting and immunofluorescence staining with antibodies to the amino terminal region of HSP90 and the extracellular domain of prolactin receptor. HSP90 β was detected highly at spermatogonial stage and in both the membrane and cytosol fractions of germ cells, while prolactin receptor was expressed in the membrane fraction of only germ cells, suggesting co-localization and co-operation of HSP90 β and prolactin receptor in the execution of spermatogonial apoptosis induced by prolactin. To investigate how HSP90 β participates in the apoptosis, we examined the interaction of HSP90 β with prolactin receptor by co-immunoprecipitation with an antibody to prolactin receptor from the organ cultures of newt testis treated with or without prolactin. HSP90 β was demonstrated to associate constitutively with prolactin receptor on the plasma membrane of germ cells, suggesting that prolactin receptor is also one of the client proteins for HSP90 β that was newly identified from newt testis. Because the association of HSP90 β with prolactin receptor implied their functional relevance, we examined the effect of an inhibitor of HSP90 β function geldanamycin, on prolactin-induced apoptosis. Dissociated testicular cells were reaggregated and cultured for 2 days in the absence or presence of prolactin or/and geldanamycin to evaluate apoptotic cells with a crescent-like nucleus stained by eosin and hematoxylin and with a TUNEL positive nucleus. Treatment of the cultures with prolactin or geldanamycin induced apoptosis. In the cultures treated with prolactin and geldanamycin, inhibition of HSP90 β function by geldanamycin was shown to potentiate prolactin-induced spermatogonial apoptosis synergistically, suggesting that the apoptosis is mediated by a defect in HSP90 β unction. Taken together, these results suggest that HSP90 β is involved in signaling prolactin-induced apoptosis through the receptor. Therefore, we speculate that, like geldanamycin, prolactin could inhibit or/and change HSP90 β function, causing the changes in varieties and statuses (i.e. phosphorylation) of the client proteins for HSP90 β interacted with prolactin receptor to induce apoptosis in the 7th generation of spermatogonia.

P36: HSP90 β , prolactin receptor, prolactin, apoptosis, spermatogenesis

ULTRASTRUCTURAL ABNORMALITY OF SPERMATOZOA AND AGE-DEPENDENT LOSS OF SPERMATOGENESIS IN TRANSGENIC RAT

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We recently established a transgenic rat (1) using the construct of porcine FSH β promoter, -852/+10 bp region, fused to the Herpes simplex virus thymidine kinase (HSV-TK) gene. The transgenic rats showed the tissue-specific expression of the HSV-TK gene in the pituitary cells producing FSH, and ectopic expression of theTK gene under the control of cryptic promotor in the testes with abnormal spermatogenesis, resulting male infertility. In this study, histology, immunohistochemistry, and electron microscopy techniques were employed to disclose the causes for the infertility in transgenic male.

Immunohistochemical analysis of HSV-TK and WT1 proteins showed that the TK protein is located in the postmeiotic germ cells, but not in spermatogonia, spermatocyte and Sertoli cells. Evaluation of spermatogenesis with electron microscopy demonstrated that both normal germ cells development and ultrastructural abnormality of testicular spermatozoa were present in the transgenic rats. In addition, age-dependent loss of spermatogenesis has also been found when compared different age of transgenic and normal rats.

The accumulations of the HSV-TK protein in the postmeiotic germ cells not only directly disrupt the spermiogenesis, but also indirectly damage the spermatogenesis. Since the Sertoli cell plays an essential role in development of spermatogenesis, those functions are affected by interaction with germ cells. Thus, this transgenic rat may be a useful animal model for studies on germ cell-Sertoli cell interaction and male infertility.

(1) Li-yi Cai *et al.* *J. Reprod. Dev.*, In press.

P37: HSV-TK, Infertility, spermatogenesis, spermatozoa, Transgenic rat

ANALYSIS OF TESTIS-SPECIFIC SERINE PROTEASES, TESSP-3 AND TESSP-4 IN MOUSE TESTES: COMPARISON WITH OTHER TESSPs

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The testis is a reproductive organ that serves two crucial functions: synthesis and secretion of steroid hormones and production of spermatozoa. Spermatogenesis is a complex process that involves formation of spermatogonia from germline stem cells; spermatogonial renewal and differentiation into primary spermatocytes; meiosis, by which diploid spermatocytes develop into haploid spermatids; and spermiogenesis, in which round spermatids mature into spermatozoa (1). Various germ-specific serine proteases have been reported in previous studies and thought to play important roles during spermatogenesis. We are particularly interested in genes encoding intracellular or extracellular proteolytic enzymes.

We cloned cDNAs for novel serine proteases from a mouse testis cDNA library by RT-PCR using degenerate primers directed at conserved sequence motif within the catalytic region of serine proteases. As a result, four novel serine proteases were isolated and designated as testis-specific serine protease-1 (TESSP-1), TESSP-2, TESSP-3 and TESSP-4. Transcripts of these clones were restricted in the testis as observed by Northern blot analysis. The clones for TESSP-1 (1,128bp), TESSP-2 (1,595bp), TESSP-3 (1,995bp) and TESSP-4 (1,839bp) were found to code for proteins of 323, 336, 383 and 373 amino acids, respectively. *In situ* hybridization analysis revealed that TESSP-1 mRNA was expressed in type B spermatogonia and spermatocytes (2), TESSP-3 mRNA was in spermatocytes and spermatids whereas mRNA of TESSP-2 and TESSP-4 were mainly expressed in spermatocytes. Immunostaining analysis showed that TESSP-1 was localized in Golgi apparatus, TESSP-2 was in ER and TESSP-3/TESSP-4 were in subcellular membrane structures. These results suggest that TESSPs play different roles during spermatogenesis in the mouse testis.

(1) Baarends WM and Grootegoed JA. (2003) Male gametogenesis, In: Fauser BCJM, (ed.), *Reproductive medicine*. Parthenon Publishing, London, pp. 31–399.

(2) Takano, N. *et al.* (2004) *Mol. Reprod. Develop.*, **70**, 1-10.

P38: protease; TESSP; spermatogenesis; testis; mouse

DELAYED DEVELOPMENT OF THE TESTIS IN GROWTH-RETARDED (*grt*) MICE

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Growth-retarded (*grt*) mice are congenitally hypothyroid and exhibit an autosomal recessive pattern of inheritance (1). In *grt* mice, the plasma level of thyroxine is significantly lower but that of thyroid-stimulating hormone (TSH) is significantly higher than those in control mice (1, 2). Our previous works suggested that a defect might exist in TSH receptor-mediated signaling, including the expression and function of TSH receptor and TSH receptor-guanine nucleotide-binding protein coupling in the thyroid gland (2, 3).

During holding *grt* mice in our animal facility, we noticed that male mice are infertile at the young adult stage (~13 weeks) but fertility is partially restored after 26 weeks-old. To understand the delayed maturity of testis in *grt* mice, we assessed the developmental pattern of their testes compared with normal mice. The testicular weight of *grt* mice was significantly slighter than age-matched normal mice until 8 weeks-old but was comparable at 13 and 26 weeks-old. The epididymis of *grt* mice was smaller before 13 weeks-old but similar to normal mice thereafter. While normal mice had mature sperms in both testes and epididymis at 5 weeks-old, any mature sperms were not seen in neither seminiferous tubules nor epididymal duct, but a lot of degenerative germinal cells were noted in the latter in 5 weeks-old *grt* mice. Mature sperms were detected after 8 weeks-old in *grt* mice. Both diameter and area of seminiferous tubules in testes of *grt* mice were significantly smaller than those of normal mice before 13 weeks-old, but they were comparable at 26 weeks-old. Additionally, the number of Leydig cells in *grt* mice was significantly smaller than those of normal mice until 8 weeks-old, but they were comparable after 13 weeks-old. The findings in this chronological study indicated that male *grt* mice might need more than 13 weeks to develop structurally mature testis. Further studies on sperm function are in progress.

(1) Yoshida, T. *et al.* (1994) *J. Endocrinol.*, **142**, 435-446.

(2) Kobayashi, K. *et al.* (2001) *Zool. Sci.*, **18**, 955-961.

(3) Kobayashi, K. *et al.* (2005) *Congenit. Anom.*, **45**, 89-92.

P39: growth-retarded mouse, hypothyroid, testis, seminiferous tubule, sperm

IDENTIFICATION OF GONADOTROPIN PRODUCING CELLS IN THE ADENOHYPOPHYSIS OF BROWN HAGFISH, *PARAMYXINE ATAMI*

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The hagfish is the extant representatives of the oldest class of vertebrates, Agnatha (Jawless vertebrates). Not only the external body features but also the pituitary gland remains in very primitive conditions. For instance, hypophysectomy in hagfish (*Eptatretus stouti*) did not show any changes in gonadal or thyroidal functions (1), and thus it remains enigma whether adeno-hypophysial hormones similar to those of more advanced vertebrates are present in the hagfish pituitary or not. However, our recent study has revealed that immunoreactive gonadotropin (GTH)-like material was demonstrable in the pituitary gland of brown hagfish, *Paramyxine atami*, and further that accumulation of GTH-like material was strongly correlated to the degrees of the developmental conditions of gonads (2). It was suggested that GTH is present in the hagfish pituitary gland. The present study aimed to identify a GTH molecule and its cellular activities in the adeno-hypophysis of brown hagfish.

First, we constructed cDNA library from pituitary of brown hagfish, and isolated two kinds of pituitary-related genes using an expressed sequence tag analysis. Based on their putative amino acid sequences, especially the positions of cysteine (Cys) residues, and molecular phylogenetic analyses, the isolated two clones were considered to be cDNAs encoding the α - and β -subunits of hagfish GTH. Hagfish GTH β contained 12 Cys residues at the homologous position to gnathostome GTH β and showed 30-43% sequence identities. On the other hand, hagfish GTH α contained 8 out of 10 Cys residues at the homologous positions to gnathostome GTH α and showed 39-45% sequence identities. In phylogenetic trees, α - and β -subunits of hagfish GTH are far removed from those of gnathostomes, and take positions as out groups, respectively. Thus, it seems most likely that there is a single GTH molecule in the hagfish representing an ancestral form that has given rise gnathostome GTHs.

Secondly, we examined the localization of GTH producing cells in the adeno-hypophysis of brown hagfish using immunohistochemical technique and *in situ* hybridization. Specific antisera were raised against the synthetic peptides of hagfish GTH α -subunit, putative amino acid positions 58-74, and β -subunit, positions 86-99. As a template for *in situ* hybridization, the hagfish GTH α -subunit cDNA (nt 69 to nt 358; 290bp) was amplified by PCR, generated the digoxigenin (DIG)-labeled RNA probes. Morphological explorations demonstrated both GTH α - and β -subunits were completely synthesized in the same cells of hagfish adeno-hypophysis. These cells were well accordance with our previously observed GTH-like cells which were identified with an antiserum raised against ovine LH β -subunit. Moreover, cellular activities of GTH-producing cells showed positive correlation with gonadal conditions of hagfish; low expression in juveniles and high in adults possessing well-developed gonad. Therefore, our present results clearly demonstrated that brown hagfish has a GTH molecule, which is an ancestral molecule of gnathostome GTHs, and it has crucial functions in the reproductive endocrine system of hagfish.

(1) Matty, A. J. *et al.* (1976) *Gen. Comp. Endocrinol.*, **30**, 500-516.

(2) Miki, M. *et al.* (2006) *Gen. Comp. Endocrinol.*, **148**, 15-21.

P40: Gonadotropin; Hagfish; Adeno-hypophysial hormones; Agnatha; Pituitary gland

IDENTIFICATION OF GROWTH HORMONE-RELEASING HORMONE, SOMATOSTATIN AND GROWTH HORMONE IN ELASMOBRANCH, DOGFISH, *Triakis scyllium*

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The hypothalamus-pituitary-liver endocrine axis plays an important role in growth regulation in fish as well as in mammals. In fish, growth hormone-releasing hormone (GHRH) and somatostatin (SS) in the hypothalamus, and growth hormone (GH) in the pituitary have been identified in a wide variety teleost fish. Biological functions of these hormones have also been characterized. In contrast to teleosts, the mechanism of growth regulation in elasmobranch is still unclear because SS and GH were identified only in ratfish (1) and blue shark (2), but GHRH is not identified yet. To get a better understanding about growth regulation in elasmobranch, we cloned GH, GHRH/PACAP, and SS cDNAs from the pituitary cDNA library of dogfish, *Triakis scyllium*, and characterized the tissue distributions of these hormones.

The pre-GH cDNA consists of a signal peptide of 27 amino acids (aa) and a mature protein of 183 aa, which was confirmed by N-terminal sequencing of isolated GH from the pituitary. Dogfish GH contains 4 cysteine residues at positions homologous to those of teleost GHs, and the residues involved in receptor binding (site 1 and 2) were highly conserved as compared with human GH (3). The GHRH precursor cDNA encoded GHRH and pituitary adenylate cyclase activating polypeptide (PACAP), the same as in teleosts. GHRH and PACAP are estimated to consist of 47 and 38 aa, based on the sequence comparison with teleosts counterparts. Dogfish GHRH showed lower sequence identity than PACAP as compared with those of teleosts. Two molecular forms of SS, SS-14 and SS-26, are derived from the precursor cDNA. Both SS-14 and 26 were highly conserved at C-terminus as compared with those of teleosts. The GH producing cells were located in the rostral pars distalis of pituitary gland, similar to that of teleosts. The PACAP immunoreactive cell bodies and fibers were located in the hypothalamus. No GHRH immunoreactive cell bodies and fibers were observed using mammalian GHRH antibody. The SS immunoreactive cell bodies and fibers were found in hypothalamus.

(1) Conlon (1990) *Gen. Comp. Endocrinol.*, **80**, 314-320.

(2) Yamaguchi *et al.* (1989) *Gen. Comp. Endocrinol.*, **73**, 252-259.

(3) Goffin *et al.* (1996) *Endocrine Rev.*, **17**, 385-410.

P41: cDNA cloning, Dogfish, GH, GHRH/PACAP, SS, Tissue Distribution

IGF-I STIMULATES SYNTHESIS AND RELEASE OF GTH IN MASU SALMON PITUITARY CELLS AT EARLY STAGES OF GAMETOGENESIS

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Insulin-like growth factor I (IGF-I) is involved in the control of reproductive function at a particular stage of sexual maturation, the onset of puberty. In salmonids, IGF-I increased gonadotropin-releasing hormone (GnRH)-stimulated release of GTH at early gametogenesis (1). IGF-I had synergistic interaction with salmon GnRH (sGnRH) to regulate GTH synthesis, but the effects were different depending on the reproductive stages (2). The aim of this study was to determine if IGF-I itself stimulates GTH synthesis and release at the early stage of gametogenesis in salmonids. Direct effects of IGF-I on expression of GTH subunit genes and release of GTHs were examined using primary pituitary cells of masu salmon (*Oncorhynchus masou*) at three reproductive stages.

Masu salmon of Mori hatchery strain were caught at early gametogenesis in February, early sexual maturation in May, and spawning in September 2003. IGF-I (0.01, 0.1, 1, 10 and 100 nM) alone or IGF-I (100 nM) and sGnRH (100 nM) were added to the primary pituitary cell cultures. Amounts of mRNAs for glycoprotein α_2 , follicle-stimulating hormone (FSH) β and luteinizing hormone (LH) β subunits were determined by real-time PCR, and levels of FSH and LH in the plasma and the culture medium were determined by RIA. In addition, the plasma IGF-I levels were determined by RIA.

In males, IGF-I alone increased three GTH subunit mRNAs on day 3 of incubation in February in dose-dependent manners. However, it did not affect the three GTH subunit mRNAs in May and September. In females, stimulatory effects of IGF-I were not observed in all stages. On the other hand, IGF-I alone stimulated release of FSH and LH in February in females, although it had no effects in males. Plasma IGF-I levels elevated from February through May in both sexes. Our results suggested that IGF-I itself has stimulatory effects on GTH synthesis and release at early gametogenesis and it functions as a metabolic signal that induces puberty in salmonids.

(1) Baker *et al.* (2000) *Biol. Reprod.*, **63**, 865-871.

(2) Ando *et al.* (2006) *Gen. Comp. Endocrinol.*, **149**, 21-29.

P42: anterior pituitary, GTH, insulin-like growth factor I, puberty, Salmon

IMMUNOHISTOCHEMICAL LOCALIZATION OF PROLACTIN-RELEASING PEPTIDE IN THE BRAIN OF THE OVOVIVIPAROUS FISH SPECIES *Poecilia reticulata* (GUPPY)

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Prolactin (PRL)-releasing peptide (PrRP) was first discovered in bovine hypothalamus [1]. In teleost fish, PrRP homologues have been isolated from brain extracts of the Japanese crucian carp, tilapia, chum salmon, and Atlantic salmon. These teleost PrRPs potently facilitate PRL secretion both *in vitro* and *in vivo*. It is also reported that intra-arterial injection of PrRP elevates PRL gene expression and plasma PRL levels in the rainbow trout, and that PrRP is essential to maintain the PRL level and osmotic balance in the goldfish. However, knowledge of the function of PrRP in fish has been restricted to certain fish species.

The guppy *Poecilia reticulata* is a euryhaline and viviparous teleost, and has been extensively used for the analysis of reproductive phenomena. It is reported that oxytocin (OXT) and vasotocin can induce premature parturition, and that gonadotropin is not involved in the induction of ovulation and parturition. Incidentally in rats, central administration of PrRP stimulates OXT release. Therefore, it is possible that PrRP regulates parturition via OXT neurons in the guppy. Thus, we examined the distribution of PrRP-immunoreactive (ir) cell bodies and fibers in the adult fish and the ontogenic development of the PrRP and PRL system by immunohistochemistry. To examine the projection of PrRP-ir fibers to OXT-ir cell bodies in the adult fish brain, double-staining immunohistochemistry was conducted using the OXT antiserum that cross-reacts with isotocin.

In adult guppies, PrRP-ir cell bodies were detected in the posterior part of the hypothalamus. In the pituitary, a small number of PrRP-ir fibers were observed adjacent to the PRL cells, whereas numerous PrRP-ir fibers were detected not only in the hypothalamus but also widely throughout the brain. PrRP-ir cell bodies and PRL cells were already detected on the birth day in the hypothalamus and pituitary, respectively. The number of PrRP-ir fibers in the brain increased as the fish developed. These results suggest that PrRP is involved in neuromodulation in the brain and that PrRP plays some physiological roles in the early development of the guppy. Furthermore, some PrRP-ir fibers were in close contact with OXT-ir cell bodies in the nucleus preopticus, suggesting that PrRP is involved in OXT release in the guppy.

(1) Hinuma *et al.* (1998) *Nature*, **393**, 272-276.

P43: prolactin-releasing peptide, prolactin, immunohistochemistry, ontogeny, guppy

**RETROGRADE AND GOLGI STAINING STUDY OF MEDAKA FISH BRAIN:
VASOTOCIN AND/OR ISOTOCIN MAY ACT AS NEUROMODULATOR**

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Previously we reported that Vasotocin/Isotocin-immunoreactive (VT/IT-ir) neurons are only present in a cluster in the nucleus preopticus (NPO) of the preoptic area in the medaka fish (*Oryzias latipes*) brain. VT/IT-ir neurons are sexually dimorphic and reduce in number after spawning in the female. Further, VT/IT-ir fibers form a plexus in the medulla oblongata (1). This plexus seems to be corresponding portion of the medulla oblongata in the midshipman which is known for vocalization of courtship behavior. In the present study, by injecting biocytin into the spinal cord of the medaka fish *in vitro*, we observed fibers extending from the diencephalon, and fibers consisting the plexus in dorsal and ventral portion of the medulla oblongata. Furthermore large neurons were labeled in the nucleus of medial longitudinal fasciculus (nMLF). The present observation is in good agreement with a previous report in the dwarf gourami (2), that the neurons of medial longitudinal fasciculus (MLF) form several different clusters, since we detected at least three kinds of cells in the MLF by golgi staining in the medaka fish brain. In the present experiment we failed to obtain any direct evidence that shows direct projection of the NPO neurons to the spinal cord. Since, the plexus in the medulla oblongata, which is labeled by the retrograde tracer applied to the spinal cord, is consisted by the VT/IT-ir fibers, it is possible that VT and/or IT detected in the plexus may play a roles as neuromodulators.

(1) Ohya and Hayashi (2006) *Zool. Sci.*, **23**, 23-29.

(2) Yamamoto *et al.* (1998) *Neurosci. Lett.*, **240**, 50-52.

P44: medaka , vasotocin, isotocin, biocytin, DiI.

IMMUNOHISTOCHEMICAL LOCALIZATION OF OREXIN PEPTIDE IN FISH BRAIN

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Orexin/hypocretin (ORX) is a neuropeptide that is involved in the regulation of feeding behavior and the sleep-wakefulness cycle in mammals (1). ORX interacts with melanophore-stimulating hormone (MSH) and gonadotropin-releasing hormone (GnRH) in mammals (2, 3). Recently it was reported that ORX is involved in food intake in goldfish *Carassius auratus* (4). Thus, it is interesting to examine whether ORX regulates food intake directly or indirectly via interacting with other neurons. However, the knowledge of the function of ORX in fish has been restricted to certain fish species. Thus, in the present study, we first examined the localization of ORX-immunoreactive (ir) cell bodies and fibers in the brain of medaka *Oryzias latipes* and masu salmon *Oncorhynchus masou* by immunohistochemistry. We further examined the interaction of ORX and MCH/MSH in medaka brain by double-staining immunohistochemistry to elucidate the neural network for food intake regulation.

ORX-ir cell bodies were detected in the nucleus posterioris periventricularis (NPPv) of the hypothalamus, and ORX-ir fibers were detected not only in the hypothalamus but also widely throughout the brain in both fish species. In medaka, some ORX-ir fibers were in close contact with MCH-ir and MSH-ir cell bodies in the hypothalamus revealed by double-staining immunohistochemistry. These results suggest that ORX is involved in neuromodulation in the brain not only in mammals but also in fish and that the neural connections between ORX and MCH/MSH neurons exist in the brain.

(1) Huesa *et al.* (2005) *J. Comp. Neurol.*, **488**, 476-491.

(2) Elias *et al.* (1998) *J. Comp. Neurol.*, **402**, 442-459.

(3) Iqbal *et al.* (2001) *J. Neuroendocrinol.*, **13**, 1033-1041.

(4) Nakamachi *et al.* (2006) *J. Neuroendocrinol.*, **18**, 290-297.

P45: Orexin, MCH, MSH, Brain, Fish

MECHANISMS OF TRH-INDUCED PRL 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*. Until now, intracellular mechanisms that regulate PRL release in amphibians and receptor structures of TRH have not been known. The aims of the present study were to clarify the contribution of extracellular Ca²⁺ on TRH-induced PRL release and to characterize the gene structures of bullfrog TRH receptors (TRHRs).

The effect of TRH on the release of PRL was studied by using a modified perfusion technique. Anterior lobes of male bullfrog (*Rana catesbeiana*) pituitary glands were continuously perfused with a medium containing TRH as a secretagogue. The amount of PRL released was measured using a newly developed homologous enzyme-immunoassay. TRH stimulated the release of PRL from perfused pituitary glands in a dose-dependent manner. In the presence of EGTA (2 mM) or Ni²⁺ (non-selective Ca²⁺-channel blocker, 3 mM), the basal PRL release was decreased and the stimulatory effect of TRH (3 x 10⁻⁹ M) on PRL release was suppressed. In the presence of nifedipine (L - type Ca²⁺-channel blocker, 10⁻⁵M), the basal PRL release was decreased. These results suggest that basal PRL release and TRH-evoked PRL release from the bullfrog pituitary depend on the extracellular Ca²⁺ and L - type Ca²⁺-channels are involved at least in the basal PRL release.

On the other hand, of TRHRs, two subtypes in some species and three subtypes in *Xenopus* have already been cloned. Here from the brain of the bullfrog, we isolated three distinct cDNAs encoding TRHR. These three subtypes were designated as fTRHR1, fTRHR2, and fTRHR3. Using RT-PCR, we investigated patterns of expressions of mRNAs of these subtypes. It was revealed that fTRHR1 was found only in the brain, fTRHR2 was in the brain and the testis, and fTRHR3 in the brain, in the anterior and, intermediate lobes, and in the testis. These results showed that three subtypes exist in the bullfrog TRHR. This is in good accordance with the result of *Xenopus* TRHRs.

P46: PRL, TRH, TRH receptor, Ca²⁺, bullfrog

EXPRESSION OF THE GLUCOCORTICOID RECEPTOR IN RAT CORTICOTROPHS DURING POSTNATAL DEVELOPMENT IS INVOLVED IN CHANGES IN PROOPIOMELANOCORTIN PROCESSING PATTERNS

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Two mammalian prohormone convertases, PC1 (also called PC3) and PC2, have been identified by cDNA cloning and shown to be involved in the cleavage of proopiomelanocortin (POMC) at paired basic amino acid sites. Previous experimental results revealed that the pattern of the proteolytic processing of POMC in the corticotrophs changes in a characteristic fashion during the postnatal period. In newborn rats, this pattern is similar to the one occurring in the melanotrophs; however, as development proceeds, this processing pattern changes to that of the adult rat, and the ability to cleave POMC into α -MSH is lost. This conversion is evidenced by the decrease in PC2 mRNA levels as postnatal development proceeds (1). Based on results with AtT-20 cells, a mouse pituitary tumor cell line, it has been suggested that PC2 mRNA is regulated by corticosterone (2).

In the present study, we examined the expression patterns of PC2 mRNA and glucocorticoid receptor (GR) in anterior corticotrophs during the postnatal period by means of immunocytochemistry with a specific antibody against the GR (3) and in situ RT-PCR analysis, respectively. Wistar male rats, aged 1, 3, and 8 weeks were sacrificed and their pituitaries fixed with 4% paraformaldehyde and cut into 4 μ m-Paraplast sections. In the 1-week-old rats, numerous PC2 mRNA-positive cells were clearly visible in the corticotrophs, whereas GR were scarcely expressed. In the 3-week- and 8-week-old rats, the number of PC2 mRNA-positive cells decreased significantly, while the number of GR-expressing cells gradually increased. In addition, the number of GR-expressing and PC2 mRNA-positive corticotrophs increased significantly as development proceeded. The expression of few GRs during the early postnatal period may be advantageous to the thymus-lymph system because corticosterone inhibits the establishment of the immune system. The steadily decreasing level of PC2 mRNA in the corticotrophs with increasing duration of the postnatal period, followed by the increase in the ratio of GR-expressing corticotrophs, initiates the secretion of a large amount of ACTH instead of α -MSH and subsequently, establishment of the hypothalamus-pituitary corticotrophs-adrenal corticosterone axis.

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(2) Day, R. *et al.* (1992) *Mol Endocrinol.*, **6**, 485-497.

(3) Ozawa, H. *et al.* (1999) *Cell Tissue Res.*, **295**, 207-214.

P47: glucocorticoid receptor, PC2, POMC, Immunocytochemistry, in situ RT-PCR

HIGHER FRACTAL DIMENSION (FD) OF C6 GLIOMA CELLS ON THE FRACTAL AKD SURFACE

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Neurons and glial cells in the brain are surrounded by a fractal environment (1, 2). Protoplasmic glial cells play a pivotal role in the CNS tissues *in vivo*, by their highly complex morphology, whereas they often present flattened fibroblast-like aspects bearing only a few simple processes on the traditional culture surface. Recent studies have implicated that cell behavior and function were affected by the topography of culture surface. However, little is known about the glial cell culture on an artificial fractal surface *in vitro*.

The previous studies showed that a fractal alkylketene dimmer (AKD) surface provided such an environment for glial cell culture (3). However, no information is available on effects of fractal surface on the complex cell morphology. In particular, the molecular mechanisms of action of the cytoskeletal gene expression remain to be elucidated.

In the present study, C6 glioma cells, a popular model in studies of glial cell morphogenesis and differentiation were therefore grown on the fractal AKD, non-fractal AKD and PLL-coated surfaces. Complexity of C6 cells was analyzed with a geometrical calculational parameter (fractal dimension, FD), and arrangements of actin and tubulin were visualized by double-fluorescent staining. The absolute amounts of actin and tubulin mRNAs were determined by quantitative real-time PCR (QRT-PCR) analyses, as an index of cytoskeletal gene expression.

A fractal AKD surface induced C6 glioma cells with higher FD and the larger numbers of copies of cytoskeletal mRNAs. The amounts of actin and tubulin mRNAs increased gradually from 0-12 h after subculture, and then γ -actin mRNA peaked off, despite continuous elevation of β -tubulin mRNA. The amounts of β -actin and α -tubulin mRNAs were kept at the same values after reaching the maximum.

In conclusion, higher FD of cells provided by fractal AKD surface was closely associated with cell differentiation. The present results from a use of fractal AKD surface further suggest that the regulation of cytoskeletal gene expression precedes appearance of morphological changes associated with differentiation.

(1) Kiselve, V.G. *et al.* (2003) *NeuroImage*, **20**, 1765.

(2) Zhang, L. *et al.* (2006) *Journal of Neuroscience Methods*, **50**, 242.

(3) Yan, H. *et al.* (2006) *Colloid Surface (A)*, **284**, 490.

P48: fractal surface, C6 glioma, differentiation, cytoskeleton, gene expression

ONSET OF METAMORPHOSIS OF CONGER EEL LEPTOCEPHALI IN LABORATORY-REARED CONDITIONS

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Eel leptocephalus larvae show drastic morphological changes, such as the forward movement of the anus and the reduction of a body length during metamorphosis. Information about the onset of metamorphosis is important for successful artificial grass-eel production; however, the mechanism of the metamorphosis is still unclear in both ecological and physiological aspects. To clarify the factors influencing the onset of metamorphosis of eel leptocephali, the conger eel (*Conger myriaster*) at the final stage of leptocephalus caught from wild were reared without feeding. The leptocephali divided into two groups by 20 days after the start of rearing: about one-third of which started metamorphosing (named “ON” individuals) and the rest remained at leptocephalus stage (“OFF” individuals), indicating that only ON individuals had been triggered for the onset of metamorphosis when they were caught. No difference between two groups (ON/OFF) was seen in the body morphology at the start of rearing but the otolith microstructure at the start of rearing that is estimated by back-calculating for 20 days using daily growth increments differed between two groups; a remarkable ring at an otoliths edge was recognized in ON individuals but not in the OFF individuals. Wild conger eel leptocephali before metamorphosis could be discriminated between ON and OFF by this otolith feature.

Metamorphosis of conger eel leptocephalus coincides with a thyroid hormone surge (1). Whole body concentrations of thyroxine (T₄) and triiodothyronine (T₃) were compared among ON and OFF phase leptocephali to investigate the hormonal regulation of the trigger for metamorphosis. The levels of T₄ and T₃ were quite low in both ON and OFF leptocephali with no significant differences. Therefore, the specific ring formation for ON individuals, which is presently the first indication of metamorphosis, may not be regulated by thyroid hormones but by other unknown physiological mechanisms.

(1) Yamano *et al.* (1991) *J. Comp. Physiol. B*, **161**, 371-375.

P49: Eel, Leptocephalus, Metamorphosis, Otolith, Thyroid hormone

CENTRAL ACTIONS OF ANGIOTENSIN II ON SPONTANEOUS BAROREFLEX SENSITIVITY IN THE TROUT *Oncorhynchus mykiss*

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Cardiovascular baroreceptor control evolved early during evolution. In teleost fish, both a reflex bradycardia and a reflex tachycardia can be observed following appropriate pharmacologically induced changes in blood pressure. In addition, in teleosts as in mammals, a brain renin-angiotensin system could be involved in central cardiovascular regulation.

The goal of the present study was to investigate the central action of native angiotensin II (ANG II) on the spontaneous baroreflex sensitivity (BRS) in unanaesthetized trout. The animals were equipped with two subcutaneous electrocardiographic (ECG) electrodes, a dorsal aorta catheter and an intracerebroventricular (ICV) cannula which was inserted within the third ventricle of the brain. The ECG and the systolic blood pressure (SBP) signals were recorded during a pre-injection period of five minutes and during five post-injection periods of five minutes. All injections were made at the fifth minute of the test. The time-series were processed with a sequence technique in order to detect the sequences of three or more consecutive increases in the SBP pulse, or three or more decreases in the SBP pulse correlated respectively with one delay beat increase of the RR interval of the ECG signal or shortening of this interval. The slope of the average regression line between the SBP and the RR intervals for each type of sequence was taken as a measure of the spontaneous BRS.

Compared with pre-injection values, the ICV injection of vehicle (0.5 µl) had no effect on heart rate (HR), SBP, the total number of positive or negative sequences or on the spontaneous BRS during the post-injection periods. By contrast, ANG II at doses of 5 and 50 pmol increased HR but only 50 pmol ANG II elevated SBP. For all doses, ANG II depressed the spontaneous BRS, but the peptide had no effect upon the number of each baroreflex sequences.

This study determines for the first time the spontaneous BRS in a non mammalian species and demonstrates an inhibitory action of ICV injection of ANG II upon this variable.

P50: Baroreflex, Angiotensin II, Fish Brain, Cardiovascular system, Signal processing

THE DUAL MODE OF ACTION OF CORTISOL ON CELL TURNOVER OF OSMOREGULATORY ESOPHAGI IN EURYHALINE FISHES

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In the seawater (SW)-acclimated euryhaline fishes, the ion/water permeability of the gastrointestinal tract is generally greater than that of freshwater (FW)-acclimated fish. The esophageal epithelium of SW fishes is simple columnar in form, whereas that of FW fishes is stratified. We have previously shown that esophageal epithelium of a euryhaline goby and tilapia displays elevated cell proliferation in FW fish, but undergoes apoptosis during SW acclimation (1). *In-vivo* cortisol treatment of the goby stimulated not only the apoptosis but also cell proliferation in the esophageal epithelium, whereas 11-deoxycorticosterone, the putative teleostean mineralocorticoid (2), did not have any impact on cell proliferation or apoptosis (3). To understand the possible dual mode of action of cortisol on the esophageal cell turnover, we have developed a method of culture tissue explants of the esophagus from euryhaline medaka (*Oryzias latipes*). Oligonucleotide detection assay and an oxidation-reduction indicator were used as indicators of apoptosis and cell proliferations, respectively. Addition of cortisol (10 nM) to the culture medium for 8 days stimulated apoptosis in the medaka esophagus, as a well-established glucocorticoid function. No effects were seen at higher doses (100 and 1000 nM). On the other hand, addition of cortisol (1000 nM) for 8 days induced the cell proliferation. The response of cell proliferation was dose-dependent within physiological range (10-1000 nM). Thus, it is likely that cortisol induces directly not only apoptosis but also cell proliferation at the esophagus in euryhaline fishes, possibly via glucocorticoid receptor, since glucocorticoid receptor shows lower sensitivity for cortisol than mineralocorticoid receptor (2).

(1) Takahashi *et al.* (2006) *J. Comp. Physiol. B*, **176**, 463-468.

(2) Prunet *et al.* (2006) *Gen. Comp. Endocrinol.*, **147**, 17-23.

(3) Takahashi *et al.* (2006) *Life Sci.*, **79**, 1873-1880.

P51: Cortisol; Apoptosis; Cell proliferation; Esophagus; Osmoregulation

PHYSIOLOGICAL ROLE AND HORMONAL REGULATION OF UREA TRANSPORTER EXPRESSED IN THE URINARY SYSTEM OF THE MARINE TOADS, *BUFO MARINUS*

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Anuran amphibians except for aquatic species usually utilize urea as a major end product of nitrogen metabolism. However, they accumulate a large amount of urea in their body fluids and maintain hyper-osmolality to tolerate a severe dehydration under dry and hyper-saline environments. To clarify the mechanisms of urea retention and effects of arginine vasotocin (AVT) on urea reabsorption in the urinary system, we characterized urea transporter isolated from the toad kidney and examined the effect of AVT on urea uptake in the cells isolated from the urinary bladder.

Bufo UT cDNA cloned from the kidney encodes a 390 amino acid residue protein, which belongs to homologue of mammalian UT-A2. The *Bufo* UT mRNA and protein were abundantly expressed in the kidney and urinary bladder, but not in the skin. When expressed in *Xenopus* oocytes by microinjection of the *Bufo* UT cRNA, the *Bufo* UT induced more than 10-fold increase in [¹⁴C]urea uptake compared with water-injected control oocytes. Phloretin, urea transport inhibitor, fully inhibited the increase of the urea uptake. When toads were acclimated to dry and hyper-saline environments for 7 days, the plasma urea concentration and circulating AVT level were significantly elevated, and these physiological changes were correlated with significant increases in the levels of *Bufo* UT mRNA in both the kidney and urinary bladder. In the epithelial cells isolated from the toad urinary bladder, addition of different AVT concentrations (10^{-12} to 10^{-8} M) to normal medium increased the urea uptake in a concentration-dependent manner. To examine the relationship between the *Bufo* UT protein expression and an increase of urea transportability, we analyzed time course of the *Bufo* UT expression levels and urea uptake in the cells treated with 10^{-8} M AVT. Treatment of 10^{-8} M AVT increased the urea uptake in the cells after 24 and 48 h incubation, but not after 12 h. According to the immunoblot analysis, UT protein expression was coincident with the results of urea uptake in the AVT-treated cells.

These results suggest that the *Bufo* UT contributes to urea reabsorption in the urinary system in response to hyperosmotic stresses and an increase of circulating AVT level. AVT seems to play an important role for mechanism of urea retention in terrestrial amphibians.

P52: Urea transporter (UT), Urea, AVT, Kidney, *Bufo marinus*

WATER PASSAGEWAY BY AVT-DEPENDENT AND -INDEPENDENT AQUAPORIN (AQP) IN SEVERAL WATER-PERMEABLE EPITHELIAL CELLS IN THE OSMOREGULATORY ORGANS OF THE TREE FROG

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An aquaporin (AQP) was cloned from a cDNA library constructed from the urinary bladder of *Hyla japonica*. This AQP (Hyla AQP-h3BL) consisted of 292 amino acid residues with high sequence homology to mammalian AQP3 (ca. 80%). The predicted amino acid sequence contains the two conserved Asn-Pro-Ala motifs found in all major intrinsic protein (MIP) family members and six putative transmembrane domains. The sequence also contains a potential N-glycosylation site at Asn-141. In a swelling assay using *Xenopus* oocytes, AQP-h3BL cRNA-injected oocytes developed a tenfold higher permeability to water than oocytes injected with water. Immunofluorescence staining using an antipeptide antibody (ST-184) against the AQP-h3BL protein revealed the presence of immunopositive cells in the basolateral plasma membrane of granular cells in the ventral pelvic and dorsal skins and in principal cells in the mucous glands. In the kidneys, immunopositive cells were found in the basolateral membrane of the principal cells in the collected ducts and a portion of the late distal tubules. The principal cells in the urinary bladder were also stained in the basolateral membrane. Both the homology of amino acid sequence and the immunostaining of the basolateral plasma membrane in water-permeable epithelial cells imply that this AQP-h3BL is a homolog to mammalian AQP3. In the ventral pelvic skins and urinary bladders, water enters into the cytoplasm through the apical plasma membrane at sites where AQP-h2, either in association with AQP-h3 or alone, is located in response to stimulation of arginine vasotocin (AVT) (1). A second AQP, AQP-x5, has also been shown to be expressed in the apical membrane of principal cells in the mucous glands (2). However, to date the water exit site for this AQP remains to be clarified. In the present study, we found specific AQP expression in the basolateral plasma membrane of the water-permeable epithelial cells, as noted above. This finding suggests that AQP-h3BL is formed for the exit of water from the cytoplasm to the surrounding capillaries in the granular cells in ventral skins and urinary bladders, whereas in the mucous glands, water enters via this AQP-h3BL and exists through AQP-x5. However, we have not yet identified any AQPs at the apical plasma membrane in the renal collected duct. In addition, this AQP-h3L protein was observed to be expressed in the plasma and nuclear membranes and some parts of cytoplasmic structures of gonadotropin (GTH) cells in the pituitary glands. This latter finding is very interesting in terms of the expression of AQP-h3BL protein, and future studies will be necessary to clarify the physiological function of the AQP in the pituitary glands.

1. Hasegawa, T. *et al.* (2003) *Endocrinology*, **144**, 4087-4096.
2. Kubota, M. *et al.* (2006) *J. Exp. Biol.*, **209**, 3199-3208.

Key words: aquaporin, vasotocin, basolateral plasma membrane, exit of water, *Hyla japonica*

ELECTROPHYSIOLOGICAL ANALYSIS OF ATRIAL NATRIURETIC PEPTIDE ACTION ON NA CHANNELS IN EPITHELIAL CELLS FROM THE URINARY BLADDER OF JAPANESE TREE FROG, *HYLA JAPONICA*Toshiki Yamada¹, Shohei Kitani², Kouhei Matsuda¹ and Minoru Uchiyama¹¹Department of Life and Environmental Science, Graduate School of Science and Engineering, University of Toyama, Japan.²Department of Biology, Faculty of Science, University of Toyama, 3190 Gofuku, Toyama 930-8555, Japan.

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, Na⁺ transports activated by ANP and cGMP were investigated electrophysiologically using a cell-attached patch-clamp technique in primary culture epithelial cells from the urinary bladder. The channel exhibited a low conductance for inward currents of 4.8 ± 0.2 pS, long open and closed times (c.a. 200 msec), and 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 Na⁺ channels (ENaC). Addition of 10^{-9} M fANP activated and significantly increased the ENaC channel activity from 0.72 ± 0.22 to 1.88 ± 0.46 . On the other hand, mean amplitudes 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 the channel activity (NPo) from 0.56 ± 0.10 to 2.00 ± 0.33 . The addition of fANP failed to activate the ENaC under presence of 10^{-6} M amiloride. These results suggested that ANP and cGMP activated Na⁺ transport via ENaC in the epithelial cells of frog urinary bladder. On the other hand, 8-Br-cGMP failed to activate the ENaC under presence of a specific PKA inhibitor, KT-5720.

In the next experiment, we measured both cGMP and cAMP production levels after treatment of fANP on the frog urinary bladder cells using radioimmunoassay kits. Frog ANP significantly increased cGMP production, but not the cAMP production. Taken together, these results suggest that fANP activates ENaC through increases in cGMP production and activation of PKA. It is also hypothesized that PKA might be activated directly by fANP via cGMP production in the epithelial cells of frog urinary bladder.

(1) Yamada *et al.* (2006) *J. Comp. Physiol. B*, **176**, 203-212.

P54: Frog urinary bladder · Atrial natriuretic peptide · Amiloride-sensitive Na⁺ channel · Single channel recording

WHAT IS PHYSIOLOGICAL SIGNIFICANCE OF INCREASED GHRELIN LEVEL IN PLASMA?

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Ghrelin stimulates feeding in rats when injected centrally or peripherally. On the other hand, in chicks, an intracerebroventricular injection of ghrelin inhibits food intake (1,2). We examined ghrelin levels in plasma and proventriculus, the glandular portion of the avian stomach, by using a specific radioimmunoassay for acylated ghrelin, as well as the expression of ghrelin mRNA in the proventriculus after a 12-h fasting period followed by a 6-h feeding period with 6-day-old layer chicks. Furthermore, in order to examine the effect of increased circulating ghrelin on food intake, a bolus intravenous injection of chicken ghrelin was given to 8-day-old chicks, and measured feeding.

Plasma ghrelin levels were increased by 12-h fasting, but once re-fed it returned to the control value. Ghrelin mRNA and the peptide levels in the proventriculus were increased by 12-h fasting, and once re-fed the ghrelin content returned to the control level, but ghrelin mRNA levels remained high. The intravenous ghrelin injection (500 pmol) did not cause any significant changes in food intake during 120 min after injection.

These results indicate that the levels of ghrelin and its mRNA with layer chicks are altered according to the feeding state. However, unlike in mammals, an increase in circulating ghrelin does not cause the promotion of food intake in chicks (3).

(1) Saito, E.-S. *et al.* (2002) *Eur. J. Pharmacol.*, **453**, 75-79.

(2) Saito, E.-S. *et al.* (2005) *Regul. Pept.*, **125**, 201-208.

(3) Kaiya *et al.* (2006) *Domest. Anim. Endocrinol.*, In press

P55: ghrelin, chicken, fasting, refeeding

THE ROLE OF HUMAN TYPE II GnRH RECEPTOR GENE IN THE REGULATION OF CELL PROLIFERATION.

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GnRH is well known as a neuropeptide hormone, which is synthesized in hypothalamus and stimulates the pituitary gland to secrete gonadotropin. Recently, the expression of GnRH and GnRH receptor in several tissues other than pituitary have been demonstrated, and GnRH has been suggested to possess several roles as controlling cell growth, cell death, or gene expression. In our laboratory, *in vitro* assay was performed and it indicated direct effects of GnRH on cell proliferation. In DU145, which is derived from human prostate cancer, GnRH inhibited cell proliferation. On the other hand, in TSU-Pr1, which is also derived from human prostate cancer, the effect was stimulatory (1). These opposite effects are reported also in other cell lines, and the underlying molecular mechanisms are being examined in this study.

While the role of GnRH being clarifying bit-by-bit, intriguing issue has arisen from our laboratory; using RNAi method, human type II GnRH receptor gene was suggested to be functional, which was thought to be a pseudogene (2). In the experiment, colony-forming efficiency assay was employed, which was established in our laboratory to easily detect the direct effect of GnRH on cell proliferation by performing it in extremely low cell density. As there was a possibility that the effect on colony-forming efficiency does not represent the effect on cell proliferation, in this study, we directly calculated the rate of cell proliferation in normal cell culture condition and clearly confirmed the relevance of type II GnRH receptor to the regulation of cell proliferation by GnRH.

(1) Enomoto *et al.* (2004) *Life Sci.*, **74**, 3141-3152.

(2) Enomoto *et al.* (2004) *Zool. Sci.*, **21**, 763-770.

P56: GnRH, GnRH receptor, cell proliferation

COMPARISON OF SERUM SEX STEROID PROFILES BETWEEN FOUR SPINE SCULPIN *Cottus kazika* AND JAPANESE SCULPIN *Cottus sp.* SE REARED IN CAPTIVITY

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The fourspine sculpin *Cottus kazika* is indigenous to Japan, but its abundance has declined in almost all rivers. Therefore since 1988, techniques for seed production of the four spine sculpin have been developed in Fukui Prefecture. However, procurement of fertilized eggs of good quality has been highly variable because both male and female fish do not mature normally in captivity. Recently the appearance of abnormally stripped eggs, which did not fertilize in the artificial insemination, occurred in cultured broodstock females. In contrast, cultured Japanese sculpin *Cottus sp.* SE mature and spawn without abnormal gonadal development. Studies on the reproductive physiology of these species are limited and fragmentary. This study compares serum profiles of sex steroids between both *C. kazika* and *Cottus sp.* SE reared in captivity.

The serum profiles of sex steroids in rearing sculpins were monitored for a full year. In Japanese sculpins *Cottus sp.* SE, serum estradiol-17 β (E2) and 11-ketotestosterone (11KT) levels were elevated significantly in the middle of vitellogenesis and spermatogenesis, respectively. Serum 17, 20 β -dihydroxy-4-pregnen-3-one (DHP) in female and male freshwater sculpins increased during the maturation period. However, in four spine sculpins, serum sex steroids levels remained low during the annual reproductive cycle, throughout ovarian and testicular development, in captivity. These results suggest that the serum sex steroids of four spine sculpins were disturbed by the captive rearing conditions.

P57: freshwater sculpins, gonadal development, reproductive cycle, sex steroids

SEX DIFFERENCE IN THE SYNTHESIS OF 7 α -HYDROXYPREGNENOLONE IN THE BRAIN OF BREEDING NEWTSShogo Haraguchi^{1,2}, Masahiro Matsunaga³, Kazuyoshi Ukena² and Kazuyoshi Tsutsui^{1,2}¹Laboratory of Integrative Brain Sciences, Department of Biology, Faculty of Education and Integrated Arts and Sciences, Waseda University, Tokyo 169-8050, Japan.²Laboratory of Brain Science, Faculty of integrated Arts and Sciences, Hiroshima University, Higashi-Hiroshima 739-8521, Japan.³Department of Psychology, Nagoya University, Nagoya 464-8601, Japan.

It is becoming clear that steroids can be synthesized *de novo* by the brain and other nervous systems. Such steroids are called neurosteroids, and *de novo* neurosteroidogenesis from cholesterol is a conserved property of vertebrate brains. Seasonal changes in neurosteroid levels in the brain have been demonstrated in seasonally breeding wild animals, such as amphibians (1, 2) and birds (3). Recently, we identified in the newt brain a novel amphibian neurosteroid, 7 α -hydroxypregnenolone that acts as a neuronal activator to stimulate locomotor activity of breeding newts by means of the dopaminergic system (4). It is well known that locomotor activity of male newts is higher than that of females during the breeding season. To understand the sex difference in locomotor activity of newts during the breeding season, we therefore investigated sex differences in the production and concentration of 7 α -hydroxypregnenolone in the newt brain. In this study, we show that the male brain actively produces 7 α -hydroxypregnenolone during the breeding season, unlike the female brain. The concentration of 7 α -hydroxypregnenolone in the brain markedly changed during the annual breeding cycle, with a maximal level in the spring breeding season (April) in male newts. The 7 α -hydroxypregnenolone concentration in the male brain was higher than that in the female brain in the breeding seasons (April and October). In the spring breeding season, the production of 7 α -hydroxypregnenolone in the brain of breeding males was higher than that of females. We then examined the expression of cytochrome P450_{7 α} , which produces 7 α -hydroxypregnenolone from pregnenolone, in the male and female brains. Cytochrome P450_{7 α} mRNA was expressed highly in the male brain than that of the female brain in the spring breeding season. These results indicate that 7 α -hydroxypregnenolone is a key factor for the increase in locomotor activity of male newts during the breeding season. This is the first demonstration of sex differences in the production and concentration of neurosteroid in any vertebrate.

(1) Takase, M. *et al.* (1999) *Endocrinology*, **140**, 1936-1944.(2) Inai, Y. *et al.* (2003) *Brain Res.*, **959**, 214-225.(3) Lea, R.W. *et al.* (2001) *Microsc. Res. Tech.*, **55**, 12-26.(4) Matsunaga, M. *et al.* (2004) *Proc. Natl. Acad. Sci. USA*, **101**, 17282-17287.P58: neurosteroid, 7 α -hydroxypregnenolone, sex difference, newt, brain

THE EFFECT OF INCUBATION TEMPERATURE ON THE SEX STEROID HORMONE SIGNALING SYSTEMS IN THE BRAIN AND GONAD OF THE SQUAMATA SPECIES WITH TEMPERATURE-DEPENDENT SEX DETERMINATION

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Some reptilian species have no sex chromosomes and their sex is determined by incubation temperature. Recently, it has been reported that incubation temperature affected not only gonadal sex but also sexual behavior in a female leopard gecko, a lizard with temperature-dependent sex determination (1). This suggests that the brain may differentiate independently from the gonadal steroid hormones to some degree. To access this possibility, the effect of incubation temperature on the expression of sex steroid hormone-related genes was investigated in the brains and gonads of the developing leopard geckoes.

In the embryo of leopard gecko, the mRNAs of two sex steroidogenic enzymes, cholesterol side chain cleavage enzyme (P450scc) and P450 aromatase (P450arom), were expressed only in the brains and gonad. The mRNAs of sex steroid hormone receptors were also expressed in these two organs. Thus, it is suggested that in the developing leopard gecko, the brain and gonad are the main steroidogenic organs and the sex steroid hormones can affect their sexual differentiation as auto-para-crine factors. The mRNA expression levels of P450arom were not affected by the incubation temperature during development and the sex difference of the expression was observed only after hatch. In the brain, however, the effect of incubation temperature on the mRNA expression levels of steroidogenic enzymes were observed before hatch. Those of P450scc were significantly higher in the brains of the embryo incubated in male-biased temperature (32°C) than those in female-biased temperature (26°C and 34°C) during temperature sensitive period (TSP). On the other hand, the expression levels of P450arom in the developing brains, were highly dependent on incubation temperature (34°C >32°C >26°C).

These results strongly suggested that incubation temperature affect on the brain directly and the brain can differentiate independently from the gonad. Because of the difference in the patterns of the effect of incubation temperature, the roles of P450scc and P450arom are different in the brain sexual differentiation.

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P59: sex differentiation, sex steroid hormone, temperature-dependent sex determination, reptiles, brain

**CIRCADIAN CHANGE IN THE BIOSYNTHESIS OF 7 α -HYDROXYPREGNE-
NONE STIMULATING LOCOMOTOR ACTIVITY AND ITS MECHANISM ME-
DIATED BY MELATONIN IN THE BRAIN OF MALE QUAIL**

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We recently identified 7 α -hydroxypregnenolone stimulating locomotor activity as a novel avian neurosteroid in the quail brain. The presence of this neurosteroid may be a conserved property of vertebrates (1). Because locomotor activity of male birds is known to be higher than that of female birds, 7 α -hydroxypregnenolone may be involved in the increase of locomotor activity of male birds. To test this hypothesis, we therefore analyzed sex differences in locomotor activity and 7 α -hydroxypregnenolone synthesis using adult quail exposed to long-day photoperiods (16L 8D; lights on at 0700 h). Behavioral analysis revealed that locomotor activity of males was much higher than that of females from the starting of light on until noon. Locomotor activity of males decreased thereafter and reached to a lower level similar to females. Both the biosynthesis of 7 α -hydroxypregnenolone and expression of cytochrome P450_{7 α} , a steroidogenic enzyme of 7 α -hydroxypregnenolone production, in the male diencephalon markedly changed during the observed 24 h-period, with a maximal level at 1100 h when locomotor activity of males was high. In contrast, locomotor activity of females and 7 α -hydroxypregnenolone synthesis and P450_{7 α} expression in the female diencephalon were constantly low during the same observed 24 h-period. Thus, 7 α -hydroxypregnenolone may be involved in the higher locomotor activity of male birds. Subsequently, we investigated the mechanism that regulates the circadian change in 7 α -hydroxypregnenolone synthesis, in male quail. Pinealectomy (Px) combined with orbital enucleation (Ex) (Px plus Ex) increased 7 α -hydroxypregnenolone synthesis in the diencephalon of males. Melatonin administration to Px plus Ex males caused a decrease in 7 α -hydroxypregnenolone synthesis in the male diencephalon. These results indicate that melatonin is a key factor for circadian change in the biosynthesis of 7 α -hydroxypregnenolone in male birds.

(1) Matsunaga, M. *et al.* (2004) *Proc. Natl. Acad. Sci. USA.*, **101**, 17282-17287.

P60: quail, 7 α -hydroxypregnenolone, melatonin

INVOLVEMENT OF NEUROPEPTIDE IN THYROXINE SURGE IN SMOLTING KOKANEE SALMON

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Involvement of thyrotropin-releasing hormone (TRH) in secretion of thyroxine (T₄) through the release of thyroid-stimulating hormone (TSH) from the pituitary is well established in mammals and birds. Prior to downstream migration, a sharp increase of plasma thyroxine (T₄ surge) has been observed in many species of salmonid fishes. However, the hypothalamic regulation in thyrotropin secretion and subsequent thyroid activity in teleost fish still remains obscure. Neuropeptides (NP) such as TRH(1), gonadotropin-releasing hormone (GnRH) (2), growth hormone-releasing hormone (GHRH)(2), and corticotrophin-releasing hormone (CRH) (3) have been proposed as potential candidates for the hypothalamic regulation of TSH. The objective of the present study was to investigate which NP is involved in the T₄ surge during smoltification in salmonid fishes.

To assess the sensitive period of T₄ secretion by environmental stimuli, underyearling kokanee salmon (*Oncorhynchus nerka*) were exposed to clay-suspended water under natural photoperiod from March to September (4). On the other hand, mTRH, oCRH, sGnRH, or hGHRH were injected intraperitoneally (IP) and intracerebrally (IC) at doses of 1µg/gBW and 1µg/µl/fish, respectively. The blood was collected from peduncle 3 hours after administration to determine the plasma T₄ concentration by time-resolved fluoroimmunoassay (TR-FIA).

Average plasma T₄ levels of control (intact) fish increased from 3.8 ng/ml in March to 9.7 ng/ml in mid June followed by a decrease in July, suggesting the mid June is a peak of smoltification. Exposure to the clay-suspended water caused distinctive T₄ surges (11 – 23 ng/ml) from April to July. While IP injections of all NP did not stimulate T₄ secretion throughout the experimental period, IC injections of GHRH or CRH caused significant increases in plasma T₄ (23 – 37 ng/ml) from April to June. These results suggest that GHRH and/or CRH have a stimulatory effect on T₄ surge via TSH in the pituitary of smolting kokanee salmon.

(1) Eales, J.G. and Himick, B.A. (1988) *Gen. Comp. Endocrinol.*, **72**, 333-339.

(2) Pahar, I. S. and Iwata, M. (1996) *Neurosci. Res.*, **26**, 299-308.

(3) Larsen, D. A. *et al.* (1998) *Gen. Cmp. Endocrinol.*, **109**, 276-285.

(4) Iwata, M. *et al.* (2003) *Aquaculture*, **222**, 315-189.

P61: Neuropeptide, Thyroxine, Salmon, Smolt

ELEVATION OF THE PITUITARY CONTENTS OF GONADOTROPINS AND PLASMA LEVELS OF FOLLICLE-STIMULATING HORMONE IN CHUM SALMON PRIOR TO INITIATION OF SPAWNING MIGRATION FROM THE BERING SEA

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The hypothalamus-pituitary-gonadal axis is a neuroendocrine system through which homing salmon concordantly regulate gonadal maturation and migratory behavior to the spawning ground. A line of previous evidence in aquacultured-salmon indicated that the pituitary-gonadal axis (PG-axis) was activated from late spring through summer, when wild chum salmon initiate spawning migration (1). In the present study, we examined whether similar activation occurs in the PG-axis of pre-migratory chum salmon in the Bering Sea.

Chum salmon were caught along the 180°-longitude line in the Bering Sea in summer and autumn from 2001 through 2003. We referred immature fish as stage I, and maturing fish as stage II and III, on the basis of the gonadosomatic indices (GSI) and histological aspects of gonads. The amounts of FSH and LH in hemisected pituitaries were determined by radioimmunoassays (2), and GTH subunit mRNAs by quantitative real-time polymerase chain reactions. Plasma levels of sex steroid hormones, such as testosterone (T), 11-ketotestosterone (11KT) and estradiol-17 β (E2), were determined by enzyme immunoassays. Mitochondrial DNA haplotypes of the fish were determined by DNA microarray (3) to distinguish the genealogical clade A (Japanese population) and clade B (Japanese, Russian, Canadian and North American populations).

The fish in the Bering Sea were comprised of those in the stage I, II and III in summer, while almost all of the fish were in the stage I in September, indicating that the stage II and III fish left for the natal river by the end of summer. The amounts of FSH and LH in stage II and III fish were 100- to 1000-fold those in stage I fish in all three years. The amounts of α 2 and FSH β mRNAs in the stage II and III fish were 2- to 10-fold, while those of LH β mRNA were about 100-fold when compared to the amounts in the stage I fish. Plasma levels of FSH in the stage II and III fish were 2- to 3-fold those of the stage I fish. Plasma levels of T, 11KT and E2 were more than 10 nM in the stage II and III fish, while about 1.0 nM in the stage I fish. Such increases were similarly confirmed in both the clade A and clade B fish.

The present results indicated that the PG-axis was activated along with the onset of spawning migration in the Bering Sea. The activation of the PG-axis at least partly by GnRH neurons should be a programmed neuroendocrine event that is inseparable from the initiation of gonadal maturation and homing behavior to the natal river.

(1) Ando, H. *et al.* (2005) *Zool. Sci.*, **22**, 379-389.

(2) Swanson, P. *et al.* (1989) *Fish Physiol. Biochem.*, **7**, 169-176.

(3) Moriya, S. *et al.* (2005) *Mar. Biotechnol.*, **6**, 430-434.

P62: chum salmon, Bering Sea, spawning migration, gonadotropin, follicle-stimulating

RELATIONSHIP OF HOMING BEHAVIOR FROM SEAWATER TO FRESH WATER WITH PLASMA LEVELS OF SEX STEROID HORMONES IN PRE-SPAWNING CHUM SALMON

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Most salmonid species migrate from ocean through the natal river for spawning. Our previous study showed that homing behavior of pre-spawning chum salmon (*Oncorhynchus keta*) in the Ishikari stock and the Sanriku stock was dependent on ocean temperature, because salmon is typical cold-water fish. There were nonetheless few lines of evidence to explain the relationship between migration from seawater (SW) to fresh water (FW) and sexual maturation. We therefore investigated a temporal profile of migratory behavior of pre-spawning chum salmon, and examined changes in plasma levels of sex steroid hormones during upstream migration from 2001 to 2005. We further investigated spontaneous behavior of fish between SW and FW environments in aquaria.

Pre-spawning fish were collected at the estuary of the Ishikari River, released after attachment of a micro data logger which can record salinity, temperature and water depth, and re-captured at the hatchery in 2005. Pre-spawning fish in the Otsuchi Bay were reared in a large aquarium, which allows fish to migrate between separated SW and FW streams. Plasma was collected from each fish every morning, and the relationship of plasma sex steroid hormones with the ratio of time spent in FW was monitored.

Pre-spawning fish of Ishikari stock continued vertical movement between SW and FW environment at the mouth of the river for about 12 hours, followed by continuous migration in FW. In aquarium experiment, plasma testosterone (T) levels in the individuals which spent mainly in FW were higher, when compared to the levels in those which stayed mainly in SW. Plasma estradiol-17_β (E2) levels in the males were lower in FW than in SW, while the levels in the females were higher in FW than SW. Plasma T levels increased during upstream migration and peaked on the midway of the river, followed by decreases at the hatchery in both sexes. The levels of E2 also reached their peaks at the estuary or on the midway of the river in all years examined.

In conclusion, migratory behavior from SW through FW is closely associated with progress in gonadal maturation. These results suggested that sex steroid hormones modulate preference of environmental salinity to FW and facilitate upstream migratory behavior.

P63: Chum salmon; Upstream migration; Sex steroid hormone; Fresh water adaptation

ISOLATION AND CHARACTERIZATION OF NEUROMEDIN U FROM THE GOLDFISH

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Neuromedin U (NMU) was first isolated from the porcine spinal cord (1). Subsequently, NMU has been identified in the central nervous system (CNS) and gastrointestinal tract of vertebrates including the human being, rat, chick and frog, and NMU can potentially act as an anorexigenic peptide in rats and Japanese quails (2-4). However, in fish, NMU has never been found, and therefore, its role is unknown. In the present study, the aims were 1) to isolate NMU cDNA from the goldfish, and 2) to examine its function. We identified four kinds of cDNAs encoding NMU precursors from the brain and intestine, suggesting that deduced NMUs with 21, 25 and 38 residues are derived from these precursors. We observed the distribution of mRNAs for NMU precursors in goldfish. The expression of these mRNAs was mainly found in CNS and intestine. Thus, the effect of feeding status upon expression of NMU precursor mRNA which is abundant in the brain and encodes NMU with 21-residue (NMU-21) was examined. Fasting for 7 days decreased significantly its mRNA expression in the brain. We synthesized NMU-21 with the C-terminal amide, and examined the effect of intracerebroventricular (ICV) administration of NMU-21 on feeding behavior in the goldfish. ICV-injected NMU-21 suppressed food consumption and locomotor activity in a dose-dependent manner. These results suggest that NMU expresses mainly in CNS and intestine, and that NMU-21 is involved in the regulation of food intake and locomotor activity in the goldfish.

(1) Minamino *et al.* (1985) *Biochem. Biophys. Res. Commun.*, **130**, 1078-1085.

(2) Kojima *et al.* (2000) *Biochem. Biophys. Res. Commun.*, **276**, 435-438.

(3) Nakazato *et al.* (2000) *Biochem. Biophys. Res. Commun.*, **277**, 191-197.

(4) Shousha *et al.* (2005) *Gen. Comp. Endocrinol.*, **140**, 156-163.

P64: neuromedin U, goldfish, mRNA, feeding behavior, anorexigenic action

INHIBITORY EFFECT OF MELANIN-CONCENTRATING HORMONE ON FEEDING BEHAVIOR IN THE GOLDFISH

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Melanin-concentrating hormone (MCH) is a hypothalamo-pituitary peptide, which had been first identified in the salmon pituitary as a hormone affecting body color (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. Our recent research has indicated that intracerebroventricular (ICV) injection of MCH influences feeding behavior in the goldfish, and may exert an anorexigenic action, unlike its orexigenic action in mammals (3). The aim of the present study was to investigate the involvement of endogenous MCH in feeding behavior in the goldfish. We examined the distribution of MCH-like immunoreactivity (MCH-LI) in the goldfish brain, and the effect of feeding status upon it. Neuronal cell bodies containing MCH-LI were localized specifically to four areas of the hypothalamus. Nerve fibers with MCH-LI were found mainly in the neurohypophysis, and a few in the mesencephalon and diencephalon. The number of neuronal cell bodies containing MCH-LI in the dorsal area adjoining the lateral recess of the third ventricle in the posterior and inferior lobes of the hypothalamus showed a significant decrease in fasted fish as compared with that in normally fed fish. We also administered an antiserum against fish MCH (anti-MCH serum) by ICV injection and examined its immunoneutralizing effect on food intake. Cumulative food intake was significantly increased by ICV injection of anti-MCH serum. These results suggest that MCH influences the feeding behavior, and that MCH can potentially function as an anorexigenic neuropeptide in the goldfish brain, unlike in mammals.

(1) Kawauchi *et al.* (1983) *Nature*, **305**, 321-323.

(2) Qu *et al.* (1996) *Nature*, **380**, 243-247.

(3) Matsuda *et al.* (2006) *Neurosci. Lett.*, **399**, 259-263.

P65: Goldfish, MCH, Anorexigenic action, Immunohistochemistry, Immunoneutralization

REGULATION OF FOOD INTAKE BY GHRELIN IN THE GOLDFISH

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Ghrelin was originally isolated from rat and human stomachs as an endogenous ligand for the growth hormone secretagogue receptor (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. It is known that peripheral ghrelin stimulates growth hormone release and appetite via the vagal afferent pathway, which is sensitive to the neurotoxin capsaicin (2) and the orexigenic action of ghrelin is mediated by the neuropeptide Y (NPY)/Agouti-related protein in mammals (3). However, in goldfish, the possible involvement of afferent pathways in mediating the orexigenic action of peripheral ghrelin, and the interactive effect of ghrelin and NPY on feeding, like that seen in mammals, have been unclear. In this study, we examined the effect of capsaicin, which destroys primary sensory (vagal and splanchnic) afferents, on the orexigenic activity induced by intraperitoneal (IP)-injected ghrelin. Pretreatment with IP-injected capsaicin cancelled the orexigenic action of IP-injected ghrelin, although IP-injected capsaicin alone did not affect food intake. We also examined the effect of intracerebroventricular (ICV) preinjection of a NPY Y1-receptor antagonist, BIBP-3226, on the orexigenic action of ICV- or IP-administered ghrelin in goldfish. Food intake induced by ICV or IP injection of ghrelin was suppressed by ICV preinjection of BIBP-3226 for 1 h. Furthermore, we studied whether either ICV or IP injection of ghrelin affects the expression of NPY mRNA in the brain using a real-time PCR method. ICV, but not IP, administration of ghrelin at a dose sufficient to stimulate food intake increased the expression of brain NPY mRNA obtained from 2 h after treatment. These results indicate that circulating ghrelin derived from peripheral tissues acts via primary sensory afferent pathways on feeding centers in the goldfish and that the orexigenic action of ghrelin is mediated by NPY pathway in the goldfish brain.

(1) Kojima *et al.* (1999) *Nature*, **402**, 656-660.

(2) Date *et al.* (2002) *Gastroenterology*, **123**, 1120-1128.

(3) Nakazato *et al.* (2001) *Nature*, **409**, 194-198.

P66: Goldfish, Ghrelin, NPY, BIBP3226, Capsaicin

EFFECT OF OCTADECANEUROPEPTIDE ON FOOD INTAKE AND LOCOMOTOR ACTIVITY IN THE GOLDFISH, *Carassius auratus*

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Octadecaneuropeptide (ODN) is a peptide derived from a precursor, the diazepam-binding inhibitor protein (DBI) in rats. DBI and its processing peptides such as ODN are also called endozepines (1). Endozepine has been identified in the central nervous system of vertebrates such as fish, amphibians, birds and mammals, indicating that endozepine is mainly distributed in the hypothalamic region (2-4). Recently, behavioral studies revealed that intracerebroventricular (ICV) injection of ODN affects feeding behavior and psychomotor activity in rodents (5,6). However, the effect of ODN has never been clarified in fish. The aim of the present study was to investigate the effect of ODN on feeding behavior and spontaneous locomotor activity in the goldfish, and we therefore examined the effect of ICV administration of synthetic rat ODN on food intake and locomotor activity using an automatic monitoring system.

ICV injection of ODN at 2.5-10 pmol/g body weight (BW) suppressed food consumption in a dose-dependent manner, and ICV-injected ODN at 5-10 pmol/g BW induced a significant decrease in the cumulative food intakes during 60 min after feeding. Locomotor activity was assessed by tracing individual swimming distances in the fish tank. ICV injection of ODN at 10 pmol/g BW induced a significant increase in locomotor activity for an observation period (60 min). These results suggest that ODN influences feeding behavior and spontaneous locomotor activity in the goldfish.

(1) Tonon *et al.* (1994) *Med. Sci.*, **10**, 433-443.

(2) Alho *et al.* (1985) *Science*, **229**, 179-182.

(3) Malagon *et al.* (1992) *Brain Res.*, **576**, 208-214.

(4) Malagon *et al.* (1992) *Peptides*, **13**, 99-107.

(5) de Mateos-Verchere *et al.* (2001) *Eur. J. Pharmacol.*, **414**, 225-231.

(6) de Mateos-Verchere *et al.* (1998) *Peptides*, **19**, 841-848.

P67: goldfish, octadecaneuropeptide, ICV injection, anorexigenic action, hypermotility

THE ROLE OF ANDROGEN IN PROTOGYNOUS SEX CHANGE OF GROUPER

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Most groupers change sex from female to male during their life time. In order to determine the role of androgen in the sex change, we first identified the sites of androgen synthesis in the gonads using immunohistological observations with specific antibody against cytochrome P45011 β -hydroxylase (P45011 β), which is the key enzyme in 11-ketotestosterone (11-KT) production, during the sex change in the honeycomb grouper, *Epinephelus merra*. P45011 β -positive cells were observed near blood vessel (BV) in the tunica enclosing the outer periphery of the ovary, but not in theca layer of the oocyte in female fish. In early and late transitional gonads, P45011 β -reactive cells appeared in the theca cells enclosing degenerate oocytes, and near the BV in the tunica ovary. However, in the testis after the sex change, immunopositive cells derived from theca cells were transformed into Leydig cells. Cell clusters near the BV in the tunica ovary also showed strong signals for P45011 β in the testis of the sex-changed male. The nuclei diameter of immunopositive cells also increased with the plasma 11-ketotestosterone (11-KT) level as the sex change progressed. These results suggest that cell clusters near BV in the tunica ovary is the location of new androgen biosynthesis, which actively produces 11-KT and plays a critical role during the sex change (1). These findings also provide evidence that the theca cells enclosing degenerate oocytes transform into Leydig cells during the sex change (2).

(1) Alam *et al.* (2005) *Cell & Tissue Research*, **320**, 323-329

(2) Alam *et al.* (2006) *Molecular Reproduction and Development*, **73**, 206-14.

Key words: Tunica ovary, androgen, sex change

FUNCTIONAL ASSOCIATIONS AMONG POSITIVE EMOTION, CENTRAL NERVOUS, PERIPHERAL AUTONOMIC NERVOUS, ENDOCRINE, AND IMMUNE SYSTEMS

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Recent studies in psychoneuroimmunology have revealed that the central nervous, peripheral autonomic nervous, endocrine, and immune systems are interrelated through complex biochemical pathways. Studies have also revealed that psychosocial stressors such as public speaking, examinations, and even short-term mental arithmetic are sufficient to bring about changes in immunological parameters. These stressors can stimulate the activities of the sympathetic nervous system and hypothalamus-pituitary-adrenal (HPA) axis, and increase circulating levels of catecholamines such as norepinephrine and epinephrine. Prolonged elevation of sympathetic nervous activity by such stressors induces extremely high levels of circulating catecholamines, reduces the number of circulating lymphocytes and activity of natural killer (NK) cells, which are a subgroup of lymphocytes that play an essential role in the cellular immune defense against virus-infected cells, bacteria or tumor cells by a β -adrenergic mechanism, and, as a result, decreases immune defense.

Then, how is it in the case of positive emotion? For example, when we look at our favorite person, our heart will be filled with fortunate feelings, and occasionally we will feel like 'flying in the sky'. Everybody knows that such romantic feeling is cheerful, strong and passionate. Accompanying romantic feeling, multiple responses in brain, peripheral autonomic nervous, endocrine and immune systems may occur. To investigate such associations among biological systems, we recorded simultaneously brain activity with positron emission tomography (PET), heart rate, blood pressure, serum levels of several hormones, and natural killer (NK) cell activity when twelve male healthy participants watched the film of their favorite persons. Interestingly, accompanying self-reported positive feelings, a component of innate immune system, NK cell activity, was activated. Various brain regions, such as the medial prefrontal cortex (MPFC), orbitofrontal cortex (OFC) and hypothalamus, were also activated. Furthermore, mean blood pressure and the serum level of prolactin, which is related to positive emotions, also increased. The activation in the OFC positively correlated with magnitudes of NK cell activity and prolactin response. This study is the first observation demonstrating that romantic feeling activates innate immune functions and also the first observation showing functional associations among romantic feeling, central nervous, peripheral autonomic nervous, endocrine, and immune systems. It is suggested that the OFC might be one of pivotal regions for top-down regulation over endocrine and immune activity accompanying romantic feeling.

P69: positive emotion, orbitofrontal cortex, innate immune system

SEX DIFFERENCE IN THE PEP-19 EXPRESSION IN SONG NUCLEI OF ZEBRA FINCHES DURING EARLY BRAIN DEVELOPMENT

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Courtship song in the zebra finch (*Taeniopygia guttata*) is produced by males but not by females. The brain regions controlling song learning and production are highly developed in males, but greatly reduced or absent in females (1) (Fig.1). The factors that initiate sexual differentiation of the brain are not known in the zebra finch. To investigate the key factors initiating sexual differentiation in brain structure, a screen was conducted for genes involved in sexually dimorphic development of the neural song system in zebra finches. cDNA microarrays were initially used to compare gene expressions in the telencephalon of hatchling males and females. We found gene expression differences between the male and female in several song control nuclei several genes showing different expressions in song control nuclei including the nucleus robustus archistriatum (RA). Interestingly, a higher expression of PEP-19 was detected in the RA of males. PEP-19 is a calmodulin-regulatory protein known to suppress the apoptotic process in PC12 cells (2). Accordingly, these results suggest that PEP-19 acts to decrease apoptosis in the RA of males during early brain development. PEP-19 may be a factor initiating sexual differentiation in the RA of zebra finches.

(1) Erhardt, J.A. *et al.* (2000) *Neuroreport.*, **11**, 3719-3723.

(2) Nottebohm, F. and Arnold, A.P. (1976) *Science*, **194**, 211-213

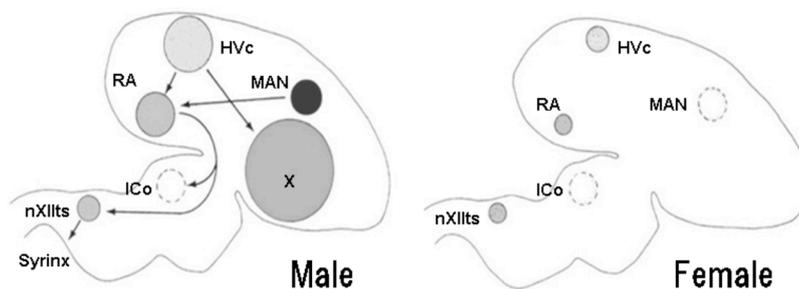


Fig.1. Sex differences in brain regions controlling song learning and production in the zebra finch [F Nottebohm & AP Arnold, *Science* (1976) 194:211-213]

P70: song nuclei, PEP-19, cDNA microarray, sex difference, zebra finch

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