Advances in Comparative Endocrinology

Vol. VII

Universitat de Barcelona

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ASOCIACIÓN IBÉRICA DE ENDOCRINOLOGÍA COMPARADA

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Advances in Comparative Endocrinology Vol. VII
Isabel Navarro, Joaquim Gutiérrez, Encarnación Capilla (eds.)

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The AIEC and the organizers of the 9th edition of the Congress (Barcelona, July 2013) offered Professors Silvia Zanuy and Manuel Carrillo, in appreciation for their important contribution to Comparative Endocrinology, a plaque of the University of Barcelona, institution where they studied the Bachelor Science in Biology and defended their Doctoral Thesis, directed by Professor José Planas Mestres.

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PRESENTATION

The present volume of the series Advances in Comparative Endocrinology contains the contributions of the participants of the 9th Congress of the "Asociación Ibérica de Endocrinología Comparada" (AIEC) that took place at the University of Barcelona in July 2013. This last edition of the meeting was a special one, since it was organized as a satellite of the 17th International Congress of Comparative Endocrinology (ICCE 2013), reflecting the current interest of the scientific community in endocrine studies using different animal models. The serial celebration of both meetings facilitated the participation in the AIEC Congress, not only of researchers from Spain and Portugal, but also of scientists coming from other European and American countries.

This volume includes a great variety of studies using vertebrate and invertebrate species, contributing with the most recent insights related to the hormonal control of important physiological processes such as growth, development, food intake, metabolism, stress and reproduction among others. The participation of prestigious and internationally recognized research groups in this latest edition of the congress made possible to reach a high scientific level in the communications presented. We are grateful to all the contributors for their active participation and the interesting discussions generated during the meeting, always in a friendly, but scientifically rigorous atmosphere. As in previous editions of the meeting, worthy of note is the presence of a high number of young scientists and students, as well as in this edition, the promotion of the visibility of women in this scientific discipline.

We would like to express our thanks for the efforts and help of the AIEC and to members of the society board for their economic support, and excellent advice on the organization of the meeting. We are also grateful to the University of Barcelona and especially to the Faculty of Biology for their help in staging the congress. We would also like to thank the organizing committee, and especially the young scientists, for their enthusiasm and dedication, making possible the successful development of the meeting. Finally, we would like to express our gratitude to "Publicacions i Edicions de la Universitat de Barcelona", for their help in the edition of the present volume.

We hope that the present joint publication of these contributions can be a renewed stimulus for the continuation and improvement of future research in Comparative Endocrinology. Our best wishes to the organizers of the next AIEC meeting in 2015 in Castellón!

The Editors

Isabel Navarro, Joaquim Gutiérrez, Encarnación Capilla

ENDOCRINE REGULATION AT THE PERIPHERY: THE EMERGING ROLE OF SKIN

D.M. Power

Comparative and Molecular Endocrinology Group, Centre of Marine Sciences, University of the Algarve, Faro, Portugal.

The skin is a frontier between the internal and external environment and an important component of innate immunity. In vertebrates, skin has a relatively well conserved organization. It is the largest neuroimmunoendocrine organ and a key interface between the endocrine, nervous and immune systems. The skin is a target of numerous endocrine factors and endocrine disorders that modify this barrier and more recently the results of next generation sequencing (NGS) experiments have substantiated this idea. For example, in mammals it is well established that adrenocorticotrophic hormone modifies skin pigmentation; estrogen changes the skin's ability to repair and thyroid hormones modify hair growth and skin moistness. In aquatic vertebrates such as fish, endocrine factors probably have similar functions, but divergence in endocrine regulation is also likely associated with the skin's specialized function as an osmoregulatory organ and also as a reservoir of minerals that are stored in scales. Recently, the idea has been emerging that skin may not only be a target for endocrine factors, but may also act as an endocrine organ. The skin is an important extra-pituitary site of prolactin (PRL) and a number of other endocrine factors that in mammals have essential functions in maintenance of the tissue's integrity. The cross-talk between the peripheral neuroendocrine system in skin and the central system is intriguing. Whether endocrine factors produced by skin contribute to systemic hormone levels has not been established yet, but their role in maintenance of skin integrity in face of a changing external environment suggests that they may have a role in contributing to systemic homeostasis.

Introduction

The skin is the largest organ in the body and has an essential role in numerous processes in vertebrates. For example it is a protective barrier; an interface between the organism and its external milieu; a thermoregulatory and sensory tissue; it is used for inter-individual communication (plumage, coloration); in addition to its central role in the innate immune response. It has generally been disregarded by anatomists and physiologists keen to reach the "interesting" organs and tissue it protects (1), although in recent years its multi-functionality and importance in innate immunity has stimulated interest. The term integument comes from the Latin *integumentum* (*integere*, from *in-* 'in' + *tegere* 'to cover') and is used to describe the outer protective layer (skin) of animals and plants (2) but also encompasses the associated appendages (scales, feathers, hairs, claws etc.). The objective of this mini-review will not be to provide an exhaustive overview of the evolution, development, structure and function of the integument and appendages in vertebrates as there are already a number of excellent reviews covering this aspect in the literature (3 and references therein). Instead, skin will

be considered first, from the perspective of a “target” for endocrine factors and secondly, as a novel endocrine organ by presenting evidence supporting this notion. A comparative approach is taken and mammalian systems are compared to teleosts. Inevitably, there is much more data available about human skin and a few representative examples and a general overview of endocrine regulation of skin will be given and the corresponding teleost examples will be considered. The functional roles of the skin will not be detailed and the paracrine/autocrine communication characteristic of immune cell regulation and the immune-endocrine link will not be explored. Instead, it is hoped that by presenting relevant examples, it will be possible to convince the reader that teleost skin should be added to the growing list of non-traditional endocrine tissue, which already includes, for example, adipose tissue and bone.

Skin Structure and Embryological Origin

The structure of human skin is well known even by the lay-man as a result of advertisements for skin care products and the impact of this sector is reflected by its value in 2010 of 10.5 billion $ (US). Strikingly, despite the temporal divide of more than 420 million years when fish and mammals shared their last common ancestor, the structure of the skin in the teleosts, the main focus of this review, is very similar to that of humans. Irrespective of the class of chordate and apparent divergence of their integuments, their development is regulated by common pathways. In all chordates, the dermomyotome, neural crest and somatopleura cells give rise to the dermis and interact with the ectoderm to form skin. In fact the formation of the neural crest, an evolutionary innovation that emerged with the chordates, generated cells with the ability to differentiate into a range of structures present in skin (e.g. sensory cells, teeth, chromatophores).

The skin in teleosts is a stratified multi-layered tissue composed of two principal layers; the outermost epidermis that is a relatively thin, avascular epithelia and the underlying thick, well-irrigated dermis that is rich in connective tissue and also contains nerves, pigment cells, adipose and muscle. The hypodermis is the interface between the dermis and the muscle and is rich in adipose cells (4). The principal differences between the skin in mammals and the majority of fish is the presence in the former of an outer keratinized water impermeable layer, rich in beta keratin, and the existence of hair follicles and hair. In contrast, all the layers of fish skin are metabolically active as there is no outer dead keratinized layer. The living epithelium is protected by a layer of mucous and mineralized scales emerge from scale pockets in the dermis (Fig. 1). Common cell types found in fish and mammalian skin include epithelial cells, basal epithelial cells, fibroblasts, melanophores, nerves and endothelial cells (blood vessels). Cells characteristic of fish skin include mucous producing goblet cells, club cells, chemosensory cells, iridophores, xanthophores and the scleroblasts and scleroclasts (associated with the scales). Several types of scale have evolved and the type a fish possesses is linked to its phylogenetic position. Advanced teleosts have elasmoid scales that are thin, flexible, transparent structures; placoid scales occur in cartilaginous fish and have a structure resembling that of a tooth; and primitive ray-finned fish have ganoid scales that consist of an osseous plate covered by a layer of dentine (4).

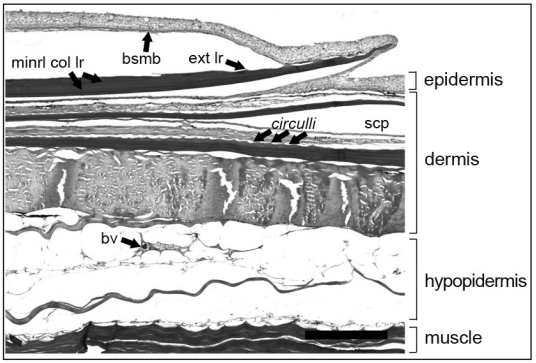

Figure 1. Longitudinal transverse sections of sea bream skin stained with Masson's trichrome. The posterior region of the elasmoid scale is orientated to the right. Connective tissue is stained green and mineralized and collagen-rich tissues are stained bright red. The three typical skin layers can be observed: the epidermis, dermis and hypodermis. Note the red staining scale with *circuli* ornamentation. Scp – scale pocket; minrl col lr – mineralized collagen layer; ext lr – external layer; bsmb – basement membrane; bv – blood vessel. The open scale pocket is a processing artifact. Scale bars: 200 μm.

The Skin as a Target of the Endocrine System

Human skin is a target for a range of hormones, namely substances secreted into the blood stream by specific ductless glands. Human skin is also influenced by paracrine factors, released by cells of the skin that act locally, but do not affect the cells that produce them and autocrine factors that act on the cell from which they are produced. In addition, evidence is accruing for a cutaneous peripheral nervous system that plays an essential role in skin homeostasis along with both sensory and autonomic inputs of the central nervous system (5). One well studied endocrine regulated process in human skin is the hair growth cycle that changes with age, stage of development and environmental factors. During the hair growth cycle, the old hair follicle is destroyed and is substituted by a newly regenerated follicle, which can produce hair of different characteristics. The main hair growth phases are relatively well characterized and most simply can be defined as anagen (the growth phase), catagen when the hair reaches its full length (apoptosis driven regression), telogen when a new hair follicle develops and exogen (hair shedding).

Light, melatonin and prolactin (PRL) are the main factors that control seasonal growth. However, specific receptors for all the major classes of hormones [steroid hormones, amino acid derivatives (e.g. thyroid hormones), peptides and proteins (e.g. vasoactive intestinal peptide, prolactin) and fatty acid derivatives (e.g. prostaglandins)] have been identified in mammalian skin (6). In some cases hormone specific receptors have been localized in specific cell types, for example receptors for parathyroid hormone and parathyroid hormone related protein and the thyroid stimulating hormone receptor are expressed in dermal fibroblasts. Several melanocortin receptors (MCR) have been identified and MCR2, which is specific for ACTH (adrenocorticotrophin) is expressed in melanocytes and adipocytes. MCR1 has high affinity for α-MSH (melanocyte stimulating hormone) and ACTH and is expressed in keratinocytes, sebocytes, sweat gland cells, endothelial cells and

cells of the immune system. MCR5 which also binds α-MSH and ACTH is present in sebocytes, sweat gland cells and adipocytes (6).

Additional factors implicated in regulation of the mammalian hair growth cycle include the sex steroids, estradiol-17β (E2) and testosterone, and the adrenal steroids. In fact, it has recently been shown that skin cells can produce glucocorticoids, androgens and estrogens and that the final steroids produced depend on the cell type or cutaneous compartment (7). The presence of steroid hormone receptors and also all the elements of the steroid biosynthetic pathways indicate that in addition to endocrine regulation, autocrine and paracrine regulation of the integument also occurs. The role of hormones in the different cutaneous compartments of mammalian skin is gradually being established.

Fish skin has received far less attention than mammalian skin and there are relatively few studies of its regulation and homeostasis. An exception is the studies of skin as an innate immune barrier, which have focussed on identifying cells responsible for the immune response; mucous compositions and bioactive compounds in mucous with protective actions. The existence and characteristic of the fish scale growth cycle has yet to be well characterized for placoid, ganoid and elasmoid scales. Nonetheless, there are a few studies reporting the dynamics of elasmoid scale regrowth after their removal (8, 9). Guerreiro et al., 2012 recently evaluated the impact of full seawater versus brackish water on scale regrowth in the sea bass (8) and showed that the bathing water did not appear to influence scale regrowth. The cycle of scale regrowth occurred in 30 days with epithelial closure occurring within 24 hours of scale removal and a visible scale appearing 3 days after scale removal. The general sea bass scale growth model for area and mass fit a linear growth curve. Since scales in fish are mobilized to release calcium and phosphorus when it is required, a number of studies in the literature have focussed on the endocrine responsiveness of scale turnover using *in vitro* scale assays. The hormones assessed in scale turnover studies are generally calcitropic (e.g. parathyroid hormone related protein (PTHrP), E2, stanniocalcin), but studies of how the fish integument is regulated by such hormones is lacking.

Recently, evidence for estrogen responsiveness of skin was demonstrated using immunohistochemistry and revealed estrogen receptor (ER) α, βa and βb are present in sea bream scales (10). Ibarz et al. (11) used proteomics to investigate the effect of E2 on the regeneration of skin damaged, by scale removal in gilthead sea bream. The results revealed that 5 days after skin damage, E2 had accelerated the rate of skin regeneration and molecular modifications were synchronized in the treated fish. Overall, the results from this proteomics study suggest that the ERs are not restricted to the scales, but are also present in other skin compartments. In fact, in a recent microarray study (unpublished), receptors for a number of different endocrine factors were identified in gilthead sea bream skin and included PRLR, GHR (growth hormone), PTH1R and thyroid receptors. A more ample collection of receptors for endocrine factors were not identified, most probably as a consequence of their absence from the microarray rather than their absence from fish skin *per se*. Characterization of the hormone responsiveness of fish skin and how hormones contribute to its homeostasis will clearly be a fruitful area of research in the future. In fact, the melanocortin receptors (MCRs) are relatively abundant in teleost fish skin and their activation by MSH and melanocyte

concentrating hormone (MCH) regulate physiological body color change. MSH and MCH, released respectively from the intermediate lobe of the pituitary and the hypothalamus, regulate pigment migration in the chromatophores in teleost fish skin when they activate their receptors and the characterization of this system and the current status has recently been reviewed (12).

The Skin as an Endocrine Organ

Vitamin D production in plants and animals is an evolutionary old process, and this is probably linked to the role of the active hormone in calcium homeostasis (13). In mammals and many other animals, production of the active hormone $1,25(OH)_2D$ from its precursor, vitamin D, occurs in several sites. The skin is one such site and makes a significant contribution to the production of the active hormone, $1,25(OH)_2D$, when the precursor in the skin is exposed to UVB radiation. Production of $1,25(OH)_2D$ by the skin is compelling evidence that in mammals it can act as an endocrine organ. There is also a growing appreciation that the dimension of the skin means that overall it may produce significant levels of other hormones; it remains to be established if hormones produced in skin have a physiologically relevant contribution to systemic hormone levels. Examples of endocrine factors that are produced ectopically by the skin include, for example, elements of the GH axis, insulin-like growth factor (IGF-I), IGF-II and IGF-binding proteins (6). POMC and its derivatives and PRL are also produced by the skin. The production of PRL by the skin is unsurprising when considered in relation to an important non-pituitary source of the hormone, the epidermal derived mammary gland. In fact, PRL is produced by several cell types in human skin, keratinocytes, sebaceous glands, fibroblasts and sweat glands and it is an important regulatory factor of the hair follicle (14). A cutaneous equivalent of the hypothalamic-pituitary-adrenal (HPA) axis has recently been identified in mammals. Corticotrophin releasing hormone (CRH) and its receptor (CRH-R1) occur in the dermal fibroblasts of human skin and stimulate the production of POMC, which is processed into ACTH, and stimulates corticosterone production. It has been argued that this cutaneous axis is triggered by the stressor (temperature, light, chemicals etc.) to which it is exposed and represents a cutaneous stress-response system, which is less evolved than the HPA axis that has cortisol as its endpoint (15). Many of the factors responsible for the cutaneous stress response in mammals arise in the highly irrigated dermis raising the possibility that it also contributes and may be synchronized with the central response, although this aspect remains to be explored.

Fish do not synthesize vitamin D, but acquire what they require from dietary sources. Until recently, it was thought that fish do not metabolize vitamin D, but several studies have revealed that they possess all the necessary enzymes for production of active metabolites, although the role of the skin remains uncertain. This contrasts with the situation in mammals and illustrates that teleost fish skin, which does not produce $1,25(OH)_2D$, cannot be considered an endocrine tissue for this hormone. Currently, in fish, there is relatively little evidence supporting a role for the skin as an endocrine tissue. Although some clues as to a possible endocrine function may be gained from studies on skin coloration. For example, when the barfin flounder (*Verasper moseri*) is reared using different background colors, POMC transcript abundance in the pituitary neurointermediate lobe (NIL) does not change, although there is an increase in circulating α-MSH (12). An in-

triguing possibility is that the melanocytes in barfin flounder skin, which produce POMC-C transcripts, may be a source of α-MSH and also supports the likely existence of a POMC cutaneous axis as found in mammals. Moreover, Des-Ac-a-MSH derived from POMC-C is measurable in the skin using high performance liquid chromatography and indicates a paracrine role for the skin. Furthermore, it may explain the increased circulatory α-MSH in barfin flounder challenged with a change in background color, which would provide further support for the skin as an endocrine tissue. More experiments will be required to test the paracrine/endocrine role of skin in control of color change and also to establish if the full endocrine axis is present and plays a role in stress as described in mammals. In fact, in fish, further studies are required to consolidate the notion of an intra-cutaneous endocrine system in fish and its potential role as an endocrine tissue.

Final Considerations

In vertebrates, numerous endocrine factors regulate the functionality of the skin in a compartment (epidermis, scale pocket etc.) and cell specific manner. Although largely overlooked, mammalian skin is an important endocrine tissue that produces an active hormone $1,25(OH)_2D$ that exerts body wide effects. More recent evidence suggests the existence of a cutaneous endocrine system, but the way in which it contributes to and is synchronized with the central endocrine axis remains to be established. The limited studies that exist suggest that the skin in teleost fish is responsive to a similar suite of hormones to those acting on human skin. However, it has not been demonstrated that fish skin produces the active hormone from vitamin D and it is suggested that a dietary source is essential. The nature of fish skin, a living epithelium that directly senses changes in the surrounding environment, raises interesting questions about the existence of a cutaneous endocrine system and its role in relating changes in the periphery to the central axis. Much work is required to demonstrate what endocrine factors are produced by skin, the cells types responsible and how they regulate skin function and homeostasis. An interesting aspect is the possible practical applications that may come from a better understanding of the skin in fish.

Acknowledgements

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FISH REPRODUCTIVE ENDOCRINOLOGY: A JOURNEY FROM BASIC TO APPLIED

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In the early seventies, marine fish culture became a reality and fish reproductive endocrinology was a broad field with a spectrum spanning from fundamental research to its zootechnical translation. This review summarizes a journey along 35 years of research in a marine teleost, the sea bass (*Dicentrarchus labrax*), which resulted in a wealth of basic information on its reproductive endocrinology, and its transfer to the aquaculture industry as practical uses and new technologies. Sea bass is a good example where basic reproductive endocrinology research has been driven by applied challenges. Since early studies on gonad morphology, seasonal changes of reproductive hormone levels and hormonal and environmental shifting of gametogenesis and spawning, novel discoveries were made on the structure/function of gonadotropins, gametogenesis/ steroideogenesis, GnRH/GnRHR and Kiss/GPR54 systems multiplicity and their functional significance, cross-communication between the components of the brain-pituitary-gonadal axis and its environmental control, puberty and sex differentiation. As we entered the genomics era, associated technologies such as recombinant hormone production and gene-transfer, among others, have been used to identify and study genes involved in reproduction and their regulation by environmental and endocrine factors. The output of this research enabled the industry to produce good quality gametes and viable juveniles on demand.

Why Sea Bass?

Sea bass was the first marine non-salmonid teleost to be commercially cultured in Europe. Presently, it is one of the most important commercial fish widely cultured in the Mediterranean. More than 2000 years ago, ancient populations used to breed sea bass since it was considered a high-value table fish. The end of the Roman Empire led to the disappearance of this type of culture and it was only in the fifteenth century that extensive, large-scale aquaculture was seen in the lagoons of the Adriatic: *vallicultura*. During the late 1960s, France and Italy competed to develop reliable mass-production techniques for juvenile sea bass and in the seventies, the French closed its biological cycle. By late 1970s the techniques were well enough developed in Mediterranean countries to provide hundreds of thousands of sea bass larvae. Despite the extensive studies on sea bass biology and breeding techniques, no information existed on the endocrine control of its reproduction, which was a major drawback to making its culture more sustainable.

The Control of the Reproductive Cycle

Sexual steroids are directly responsible for gamete production. The first RIA to determine its plasma levels in sea bass was set up in 1990 (1). It was suggested,

for the first time, that Estradiol (E2) was involved in vitellogenin (Vtg) synthesis during vitellogenesis in female and the tight correlation between plasma levels of 11Ketotestosterone (11KT) and the process of spermatozoid formation in male sea bass. It was also shown that maximum levels of sex steroid occurred under low temperature and short photoperiod conditions and that in females E2 and oocyte diameter peaked one month after the peak of 11KT in males fitting well with the difference found in the duration of the spawning span of both sexes.

In 1984, it was possible to advance or delay sea bass spontaneous spawning by modifying the cycles of photoperiod although maintaining the temperature constantly low (2). But to adjust these cycles was cumbersome and simpler methods that would provide the same results were needed. It was known from pioneer studies in trout that the absolute magnitude of daylight did not affect the timing of the spawning season, but the change of direction between both photoperiods (from long to short or vice versa) did. Also, a hypothesis on the existence of an endogenous mechanism involved in the timing of reproduction was formulated (2). Thus by using both constant light and square waves of light regimes administered at different times of the annual cycle and extending these type of studies over four consecutive years we confirmed, in sea bass, that different light cues are not required to complete spawning as this occurs equally in fish maintained under constant long or short light regimes. Therefore, in sea bass the timing of reproduction is coordinated by an endogenous rhythm entrained by the photoperiod. Hence, early exposure to long days in the sexual cycle advances spawning time whereas late exposure to long days leads to a delay of spawning. These shifts in the spawning time resulted from changes in hormonal rhythms, since the profiles of plasma E2 and Vtg were modified by exposure to long days at different times of the year, but maintaining a tight correlation as long days administered early in the year advanced the surge of E2 and Vtg, whereas a delay was observed when administered late in the year. No significant reduction was observed in egg and fry quality and fecundity of broodstock photoperiodically advanced, but poor fecundation and egg survival was seen in photoperiodically delayed fish (2). What caused this dysfunction? Previous experimental studies had shown that artificial seawater heating (>17 ºC) during fall induced a delay of the spawning time, increased ovarian atresia and decreased fecundity, while cooling (≤17 ºC) advanced the spawning time with respect to controls without affecting egg quality. Thus, in sea bass temperature is probably the factor responsible for the observed spawn disorders, as a threshold exists (17 ºC) above which either no spawning takes place or the quality of gametes is impaired. Thus, inappropriate combination of environmental cues like a mismatch between photoperiod and temperature (i.e. long photoperiod-low temperature) alters the mechanism of estrogen synthesis within the follicle modifying the rates of vitellogenesis and atresia (2). This is an important technical issue to consider in sea bass (or other fish species) hatcheries since at least inadequate combination of photoperiod and temperature is likely to be responsible for important reproductive dysfunctions (2). Afterwards, the endocrine control of sea bass oocyte maturation was accurately studied both *in vitro* and *in vivo*. A detailed correlation between oocyte development and hormonal changes through the reproductive cycle was established. Maturation inducing steroid(s) for sea bass were identified as 4-pregnen-17α,20β-diol-3-one (DHP) and 4-pregnen-17α,20β,21-triol-3-one (20βS). So DHP levels peaked in females with the most advanced oocytes at post-vitelogenesis and were associated with

the control of early oocyte maturation (e-MAT). 20βS peaked in females with the most advanced oocytes in a stage immediately prior to ovulation being ascribed to the control of final oocyte maturation (fMAT) (3). These studies also demonstrated that female sea bass may ovulate up to four times in the same spawning season experimentally confirming sea bass as fish with group-synchronous ovarian development. This type of fish require an endocrine mechanism which ensures the successive maturations and ovulations of clutches of oocytes without affecting subsequent ones in the same spawning season. Our studies in sea bass suggested that there is a mechanism of shifts in gonadal steroidogenesis which very likely are responsible for regulation of group-synchronous ovarian development, ovulation and spermiation (7, 8).

In the early 2000, the existence of two gonadotropins with similar activity of mammalian follicle-stimulating (FSH) and luteotropic (LH) hormones was made clear and in fact the three gonadotropin subunits (GPα, βI and βII) of different fish species including sea bass were cloned (4). To study the regulation of the final stages of gonadal development, sea bass LH was purified and a specific ELISA was set up. Pituitary and plasma Lh profiles exhibited a clear parallelism with gonadal growth along the reproductive cycle. Maximum Lh in pituitary and plasma was observed in February at the time of maximum ovulation and spermiation; thus it was concluded that Lh would play a major role in the regulation of maturation and ovulation (4). Afterwards, two sea bass cDNAs encoding an Fsh receptor (sbsFshr) and an Lh receptor (sbsLhr) were cloned and characterized. The expression profile of the sbsLhr during maturation-ovulation and its tight correlation with the expression of the steroidogenic acute regulatory (StAR) protein confirmed the participation of Lh in the steroidogenic shift which is typically accompanied by an increase in steroid synthesis responsible for the final stages of ovarian development (5).

In 1991, we demonstrated for the first time the presence in the brain of sea bass of a endogenous gonadotrophin-releasing hormone-like peptide (GnRH); specifically the salmon form (sGnRH; now GnRH3) forming a rostro-caudally oriented continuum of structures from the olfactory nerves to the pituitary. In 2001, three cDNAs encoding three different pre-pro-GnRHs: sGnRH, sea bream (sbGnRH, now GnRH1) and chicken (cIIGnRH, now GnRH2) were cloned in sea bass allowing the study of a detailed distribution of the cells expressing three GnRH-associated peptides (GAPs) in the brain of a single species. This gave, for the first time, unambiguous information on the distribution of projections of the three different GnRH forms. In particular it was shown that sbGAP-ir fibers reached the *proximal pars distalis* and the border of the *pars intermedia* of the pituitary, where gonadotrophic cells and GnRH receptors were also found. It was concluded that GnRH1 had an important role in the stimulation of gonadotropin secretion (6). The set up of specific inmunoassays for the three GnRHs and the analysis of pituitary profiles during gonadal recrudescence confirmed this assumption. Levels of GnRH2 and GnRH3 remained low and unchanged, while those of GnRH1 showed significant elevations during active spermatogenesis and spermiogenesis. These changes in pituitary profile of GnRH1 were associated to changes in Lh plasma profile. Thus, GnRH1 is considered to be responsible for the synthesis and release of Lh. These studies greatly contributed to improving sea bass breeding technologies which are used when broodstock spontaneous spawn does not occur or when synchronization is required (7).

Control Onset of Puberty in Intensive Fish Farming, a Necessary Issue

The abundant research effort carried out on broodstock management, husbandry, nutrition and feeding strategies of cultivated sea bass resulted in a more suitable progeny with better growth performance. As a result, a problem has emerged in its intensive culture: precocious reproduction, which seriously affects growth and reduces economic returns. Under intensive culture, ration-size male sea bass exhibit 20-40% less body weight than female at harvest time (2, 8). So, an all female monosex population seems to be the most logical solution. However, under intensive culture, some females exhibit vitellogenic ovaries in the second year of life. Also, nowadays there is an overproduction of ration-size sea bass which suggests the need for other production strategies. Thus, industries try to promote and diversify their range of products. In this context, production is strongly quality-oriented with many companies positioning their fish in the high-priced premium segment. Hence, puberty control seems a good strategy and significant efforts are currently being done in the study of the mechanism(s) responsible for the onset of fish puberty. In sea bass, early studies showed that the use of various photoperiod regimes can modify events associated with the first testicular recrudescence (8). Specifically, a constant long photoperiod resulted in a clear phase shift of the reproductive patterns, delaying onset of puberty and providing the fish with extra time to grow. Later, it was shown that long-term exposure to a continuous light (LL) regime was very effective in inhibiting gonad growth and thus occurrence of male precocity .In fact, 11KT plasma levels and expression of all three gonadotropin subunits remained low and unchanged compared to control under simulated natural photoperiod. Moreover, a series of studies directed to select a period of photolability to LL made possible to prevent the presence of male precocity by applying one month LL in September (8)**.** This methodology is easy to apply in the industry with no stressful effects as was the case with the use of LL all year around.

At this point, an obvious question is how to control puberty. Studies in salmonids show that Fsh controls early gonadal events of ovarian and testicular growth. Therefore, Fsh was purified from pituitaries of sea bass and its biochemical and biological properties studied. Later, a species specific ELISA was developed and Fsh plasma profile examined both in males and female along the reproductive cycle. Fsh plasma levels increased during testicular recrudescence and fell to baseline just before spermiation in males and gradually increased during vitellogenesis and declined at maturation-ovulation in females. This indicates that in male sea bass, Fsh is important to regulate spermatogenesis, but not spermiogenesis-spermiation and in females Fsh stimulates oocyte vitellogenic growth, but it does not seem to be directly associated with oocyte maturation and ovulation. Thus, it is very likely that Fsh is involved in the onset of puberty in both male and female sea bass (4, 5, 7, 8).

It is known that in a sibling population of prepubertal male sea bass, only the larger ones attain puberty at the first year of life. Thus, if size is important to attain gonad recrudescence, there must be a link upstream of the reproductive hormonal cascade between size-metabolic status and the choice to breed. In mammals, the hypothalamic KISS1 system is responsible for changes in the metabolic status of the organism. Moreover, a number of metabolic signals, with key roles in

energy homeostasis, such as leptin, and neuropeptide Y (NPY), have been shown to directly or indirectly influence the expression of *Kiss1* (9). Previously, we have shown an NPY-induced Lh secretion in sea bass dependent on energetic status and a direct action of leptin on pituitary Lh release enhanced by NPY in late prepubertal sea bass (2). Besides, two distinct kiss-like genes have been cloned and characterized together with their respective receptors. Functional studies demonstrated that kiss2 decapeptide was significantly more potent than kiss1 peptide in inducing sea bass Lh and Fsh secretion (9). Recent detailed information on the expression sites of *kiss1* and *kiss2* genes in the brain of sea bass undergoing sexual maturation suggests that the *kiss1* gene would participate in regulating reproduction through the mediobasal hypothalamus kiss1 population sensitive to estrogens and also through expression in the Fsh cells, although more data are needed to further substantiate this (10). A further study on the distribution of kiss2 fibers and *kiss-R2* mRNA expressing cells in the brain of sea bass indicates that kiss2 is likely to play a much wider range of potential functions since neurons expressing NPY and somatostatin are clear targets for kisspeptins (11). Thus, it is very likely that the kiss/Gpr54 is a very good candidate to be a link between metabolic status and reproduction in sea bass.

The Genomic Era and Associated Technologies

Taking advantage of the recombinant technology, dimeric (rFsh) or single-chain recombinant sea bass Fsh (scrFsh) were produced and efficiently secreted in two systems, insect (Sf9) and mammalian (CHO) cells. To study the dual role of Fsh and Lh in sea bass further, specific recombinant dimeric (rLh) and single-chain (scrLh) Lh were produced in Sf9 and CHO cells. Both rFsh and rLh stimulated their respective cognate receptors in a specific and dose dependent manner. Fsh stimulates follicular development in females and spermatogenesis in males, whereas Lh is responsible for oocyte maturation and ovulation in females and spermiation in males, both Lh and Fsh promote steroideogenesis (12). Later experiments in prepubertal male sea bass treated with scFsh demonstrated that in sea bass, Fsh promotes germ and Sertoli cell proliferation and thus is one of the early signals to proceed with spermatogenesis.The use of modified recombinant peptides is a possible alternative to solve important dysfunctions in aquaculture. But the production of such proteins is expensive and their availability limited. One potential solution is the use of gene based therapies. Very recently, we have evaluated the suitability of this technology by injecting in the muscle of mature male sea bass a plasmid containing a gene coding for a single-chain (sc) form of the Lh (12). The effect of scLh plasmid treatment was compared with that of scLh produced in mammalian cells. Both treatments resulted in an increase of plasma Lh and sperm release of equal quality, but plasmid injection provoked longer lasting and higher plasma Lh levels, compared with the injection of an scLh. Therefore, somatic gene transfer is a realistic approach in fish for hormone therapy of dysfunctions due to low hormone levels or just to synchronize spawning. Additionally, the plasmid treatment represents a better approach for dysfunctions requiring long-lasting treatments (triggering vitellogenesis/spermatogenesis, controlling or advancing onset of puberty …). New ongoing tools such as 454 transcriptomics or a specific microarray enriched in reproduction-related genes, will certainly contribute to large-scale analysis of gene expression under different conditions, which will enable a more sustainable sea bass aquaculture.

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ENTRAINMENT OF CIRCADIAN OSCILLATORS IN FISH: INTEGRATING EXTERNAL AND ENDOGENOUS SIGNALS

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The circadian system drives daily physiological and behavioral rhythms that allow animals to anticipate cyclic environmental changes. The discovery of the so-called "clock genes", which are very well conserved through vertebrate phylogeny, highlighted the molecular mechanism of circadian oscillators functioning, based on transcription and translation cycles (~24 h), of these clock genes. The study of clock genes also evidenced that circadian systems are formed by a net of oscillators distributed at central and peripheral locations. Using the goldfish (*Carassius auratus*) as a model species, we have investigated if central and peripheral oscillators are both entrained by the main environmental cues, the light-dark (LD) cycle and the feeding time, as well as how clocks in different locations can communicate among themselves. The light/dark cycle synchronizes clock gene rhythms in peripheral and central locations in this teleost. The clock gene *Per1a* anticipates the onset of the light in all studied tissues (as in zebrafish), while light-induced *Per2a* expression is found only in the retina and the hypothalamus, but not in peripheral tissues. A feeding schedule also synchronizes goldfish oscillators, peripheral tissues being more sensitive to this input. Besides environmental zeitgebers, some "endogenous entraining signals" such as glucocorticoids, orexin and ghrelin can also modify clock gene expression (mainly genes from the *Per* family) at several locations in goldfish. In spite of this new information, the main question remains unsolved, how does the fishes' circadian system integrate external and endogenous signals? Our hypothesis considers that the endogenous signals could determine sensitivity to environmental cues.

Introduction

Organisms exhibit daily rhythms in several functions controlled by the circadian system which allow them to anticipate changes in the environment. In vertebrates this system is composed of a network of oscillators located in the whole body (1, 2). The molecular core of these biological clocks is based on transcriptional-translational feedback loops (with a periodicity of about 24 h) of a set of genes called clock genes, which are well conserved in vertebrates (1, 2). Most of our knowledge about the molecular functioning of the circadian system in fish comes from multiple studies on zebrafish (*Danio rerio;* 2). However, it is unknown at present if some of the surprising properties of zebrafish circadian oscillators (all cells act as light-sensitive clocks; 2) are an exception or a rule in fish. It is also unknown how different oscillators communicate among themselves to generate an overt rhythm. Using the goldfish as a model, we have tried to tackle some of these questions.

Clock Genes Cloning and Synchronization by Photoperiod and Feeding Time in Goldfish

The cloning of some clock genes from the main loop that maintains circadian oscillations in goldfish (3, 4; Fig. 1) has led to the characterization of daily rhythmic profiles of such clock genes in central and peripheral locations in animals synchronized to different environmental cues.

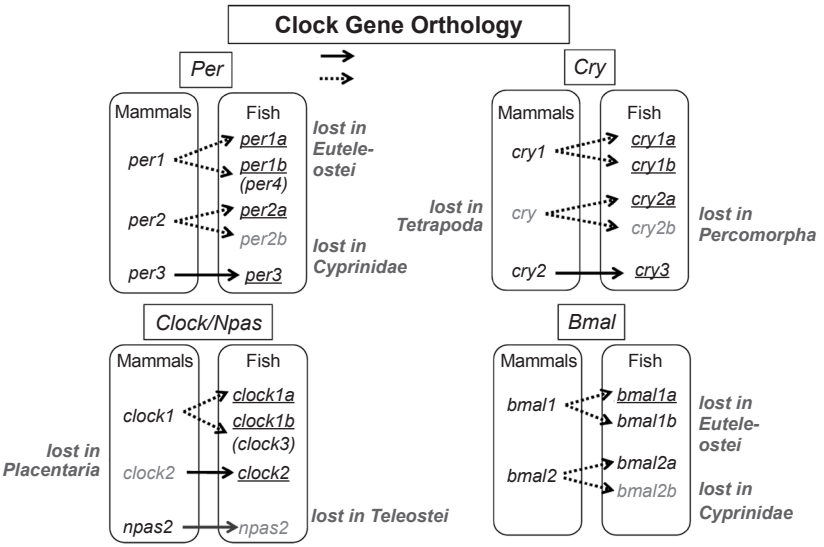

Figure 1. Comparative orthology of clock genes in mammals and fish. On line available sequences belonging to the four main families of clock genes have been used (Alonso-Gómez et al., unpublished). Continuous black arrows indicate orthology while discontinuous arrows indicate teleost-specific genome duplication. The genes underlined have already been found (partially cloned) in goldfish. Genes in gray have been lost in the corresponding group. Genes in black and not underlined have not been cloned yet in goldfish, but are expected to exist.

Regarding the negative elements, *Per1a* is considered to be a classic clock-regulated gene induced by the dimer CLOCK-BMAL1 in zebrafish (2), and it can be synchronized by either a feeding schedule or the LD cycle in the brain and peripheral locations in goldfish. However, peripheral clocks are in general more sensitive to food related cues (3-6), it is even reported that one meal could reset the clock in the liver (5). The acrophase of this gene anticipates the light onset, when a light-dark cycle is present (3, 4, 6), and food supply, under constant light and a feeding schedule (5, 6). The regulation of *gPer3* and *gCry3* expression seems to be similar to *gPer1* in goldfish central locations (3-6). However *gPer3* could be driven by the feeding schedule instead of the LD cycle when both cues are present, at least in the gut (6). Daily rhythms of *gCry1a* and *gCry2a* genes seem to be influenced mainly by the LD cycle since they are not synchronized by a feeding schedule (4, 5). Finally, it is well known that light directly induces *Per2a* expression in all zebrafish tissues (2), but in goldfish, *gPer2a* regulation differs between cen-

tral (retina and the hypothalamus) and peripheral structures, being directly regulated by light only in central ones (3-6, unpublished own results). In any case, the role of *Per2a* as the target of LD cycle to synchronize endogenous oscillators in teleosts is probably a conserved feature, at least in photosensitive tissues (2, 3, 4, unpublished own results).

The cloned positive elements (*Bmal1a*, *Clock1a* and *Clock2*) from the core loop of the clock have been less studied to date. They are rhythmic in goldfish maintained under an LD cycle, with acrophases occurring during the day, i.e. with inverted profiles respective to *gPer1a* and *gPer3* genes, in central and peripheral locations (4, unpublished own results), as expected for functional circadian oscillators. The effect of a feeding schedule on these genes has not yet been studied.

All these data support the idea that the goldfish circadian system is formed by a net of central and peripheral oscillators, most of them synchronized at least by the two main environmental cues, the LD cycle and feeding time. This agrees with the idea of a unique mechanism for circadian synchronization to different environmental cues recently proposed in mammals (1), and brings into the key question that two types of different oscillators exist, the light entrained oscillators (LEOs) and food entrained oscillators (FEOs).

Endogenous Entraining Signals

Bearing in mind that the goldfish circadian system is composed of a net of clocks, we can speculate that each oscillator functions as a complete mini-circadian system with its own outputs and inputs. In this sense, we have investigated putative molecules that could be involved, as internal time messengers, in the crosstalking among different oscillators. We studied hormones related with the LD cycle and the synchronization of peripheral oscillators, like melatonin and cortisol (7, 8). In addition, we studied two orexigenic peptides involved in food intake that could be potential signals of a feeding-fasting cycle (i.e. the signals of a feeding schedule), the central neuropeptide orexin and the peripheral ghrelin (9).

It is known that goldfish show pronounced daily locomotor activity rhythms that can be synchronized by both the LD cycle and feeding time (5). Such rhythmic activity can be used as an output of the circadian system to test the possible synchronization by the hormones of interest, when injected during several consecutive days at the same time. Under constant light (i.e. in the absence of the zeitgeber LD cycle) both melatonin and orexin synchronize daily locomotor activity rhythms in goldfish (melatonin in randomly fed animals, unpublished own data; and orexin in fasted fish, 10). Besides, the administration of a ghrelin antagonist hampered the appearance of food anticipatory activity (unpublished own data), a behavior that depends on the food entrained oscillator(s). Altogether, these results support the idea that these hormones are involved in the outputs of the goldfish circadian system, either ahead of the molecular oscillators or behind them.

On the other hand, it was investigated whether acute treatment with orexin, ghrelin or cortisol modifies clock gene expression in different locations. A central orexin administration increased the three *period* genes, *gPer1a*, *gPer2a* and *gPer3,* in goldfish hypothalamus and *gPer1a* and *gPer3* in the foregut, at 3 h post-injection.

Orexin treatment does not affect clock gene expression in the liver nor in the hindgut (Fig 2; 10). Ghrelin treatment (intraperitoneal injection) induced *gPer1a* and *gPer3* genes in the hypothalamus and liver of goldfish at 3 h post-injection (Fig. 2; unpublished own data). None of the peptides altered *gBmal1a* expression in any studied tissue. Moreover, orexin also induced ghrelin expression (~10 fold) in goldfish foregut, and ghrelin induced hypothalamic orexin expression, as previously reported (9, 10). These results indicate that these two peptides can act via their target tissues as inputs to the circadian system, and support brain-gut bidirectional crosstalking.

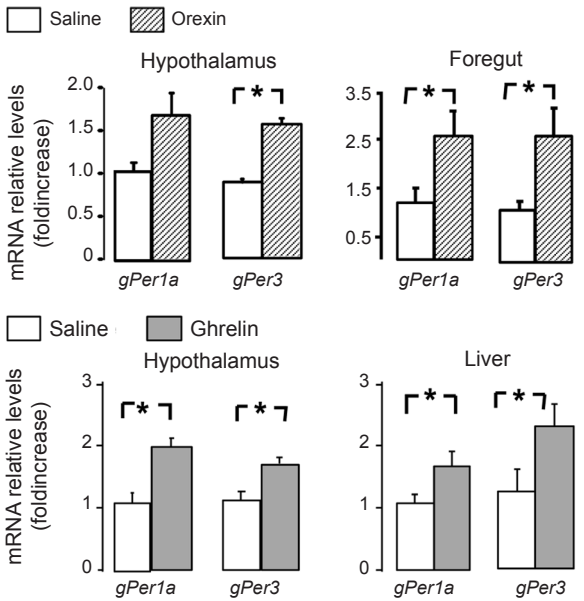

Figure 2. Effect of orexin-A (10 ng/g bw, intracerebroventricular injection) and ghrelin (100 ng/g bw, intraperitoneally injection) on clock gene expression in goldfish. The transcript relative amount is expressed as mean ± SEM (n=7). *: p<0.05 (t-student).

Finally, cortisol and its analog, dexamethasone, also induced *gPer1a* in the hypothalamus and liver of goldfish, as previously described in mammals (8, 11), suggesting a conserved mechanism of regulation for this gene (see Sánchez-Bretaño et al., this volume).

Since all these studied hormones can modify clock gene expression at different locations, we propose that some endogenous signals could determine the clock sensitivity to other inputs. The clockwork could be altered with changes in the abundance of any of the genes of the molecular core. In fact, this is the way that classical inputs synchronize the oscillators. The previously mentioned *per2*-light induction leads to an LD cycle synchronization. Similarly, endogenous signals that increase *per1a* (orexin, ghrelin, cortisol) could shift the phase of clock gene expression, and then modify the sensitivity to ulterior inputs.

Summary, Unresolved Questions and Perspectives

The study of the molecular core of circadian oscillators from a physiological point of view, i.e. taking into account the functioning of the whole net of oscillators that form the circadian system, is a recent issue in teleosts. The goldfish is a very promising model because of the availability of many valid tools. Daily locomotor activity rhythm, one of the best known rhythms of the circadian system, can be easily measured. A variety of clock genes have been cloned at central and peripheral locations. And feeding regulators are well characterized in this species. All this highlights *Carassius auratus* as being of special interest to study the synchronization of the circadian system by other inputs than the LD cycle. All the above-mentioned results obtained in goldfish are summarized in Figure 3 (see the legend for explanations).

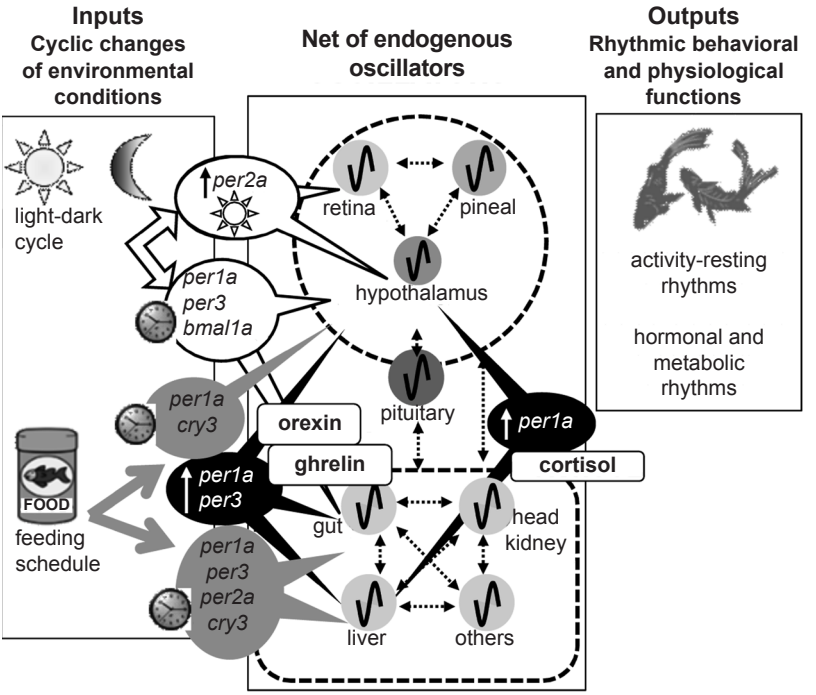

Figure 3. Summary of the goldfish circadian system organization. It consists of three main elements: the inputs, such as the LD cycle and feeding schedule; the net of endogenous oscillators that possess the molecular clockwork machinery; and the outputs or overt rhythms, such as the activity-resting daily cycle. Changes in clock genes related with the LD cycle are indicated in white balloons while changes related with the feeding schedule are showed in gray balloons. Genes on black background are modified by endogenous hormones. A clock inside the balloon implies that these genes are synchronized by the corresponding zeitgeber in the indicated locations. The arrows show the induction of some clock genes by specific signals: light increases the expression of *per2a* in the retina and the hypothalamus of goldfish; hypothalamic orexin and peripheral ghrelin induce *per1a* and *per3* in the hypothalamus, the gut (only orexin) and the liver (only ghrelin); and cortisol increases *per1a* in the hypothalamus and the liver. At present, it remains to be studied which other genes are targets for the studied hormones (square boxes), other putative entraining sig-

nals (such as the nervous system), or how the animal integrates all this information to finally generate an output.

Is despite of the recent advances regarding circadian organization in goldfish, various main questions remain unsolved (Fig. 3). It is yet unknown if extraretinal and extrapineal photoreception is widespread in other fish species as occurs in the zebrafish (our findings suggest that this is not the case in goldfish; 6). Regarding the crosstalking among oscillators, which other hormones could be involved? As in mammals (1), is the nervous system in fish acting as an input of circadian oscillators? Which other clock genes are targets of each temporal signal? And finally, how does fish circadian system integrate external and endogenous signals to give an overt rhythm?

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GHRELIN GENE PRODUCTS IN THE CONTROL OF MAMMALIAN PITUITARY FUNCTION: A SUGGESTIVE INVITATION FOR COMPARATIVE ENDOCRINOLOGISTS

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The ghrelin gene is a complex, tightly regulated and well-conserved entity that encodes for a family of peptide hormones with a wide variety of functions, including the regulation of various hypothalamic-pituitary-systemic axis functions. The ghrelin gene encompasses several coding and non-coding exons, depending on the species, which can be combined to generate a number of different ghrelin gene derived pre-pro-hormones. The most widely expressed transcript is pre-pro-ghrelin, which encodes for native ghrelin, the most well known and first identified ghrelin gene product, and obestatin. Native ghrelin can be modified by the ghrelin-O-acyl transferase (GOAT) enzyme to generate acylated-ghrelin or can remain unacylated (UAG). Interestingly, the recently identified human pre-pro-In1-ghrelin (or its pre-pro-In2-ghrelin counterpart in mice), which is produced from a special type of alternative splicing named intron retention, is more abundantly expressed in the pituitary gland than the native pre-pro-ghrelin. It is of note that all these mRNA variants and/or proteins have been shown to differentially regulate pituitary functions in several mammalian species. In particular, acylated ghrelin stimulates GH and ACTH release in primate primary pituitary cell cultures and human secreting-adenomas, but not in mouse primary pituitary cell cultures. UAG does not alter hormone secretion in any species, while obestatin stimulates ACTH release to a lesser extent than AG in all species, but inhibits GH release in primate and mouse primary pituitary cell cultures. In1-ghrelin is over-expressed in pituitary adenomas and stimulates GH and ACTH release in the species tested. These results demonstrate that acylated ghrelin is not the only product derived from the ghrelin gene able to exert a key regulatory role in pituitary gland secretions, and also, that most of the actions exerted by ghrelin gene products are conserved among species.

Introduction

Ghrelin is a 28-aa peptide initially identified by Kojima el at (1) in rats by reverse pharmacology. Indeed, the ghrelin receptor (GHSR or growth hormone secretagogue receptor) was initially discovered by its ability to bind artificial compounds with GH-releasing activity such as GHRP-6 or hexarelin (2) and years later, ghrelin was identified as the endogenous ligand for this orphan receptor. Ghrelin is a multifactorial hormone produced in a wide variety of tissues where it can exert autocrine and paracrine actions (3). Although ghrelin was discovered by its ability to stimulate GH secretion, it can also modulate the secretion and/or production of several pituitary hormones. In addition, ghrelin has been shown to exert other

functions in the CNS acting as a neuroprotective hormone and as a modulator of memory and learning processes (4). Additionally, ghrelin has been shown to be involved in a number of divergent functions such as regulation of food intake, body weight gain, insulin release and β-cell survival, adiposity, and the control of energy homeostasis (5). Likewise, the ghrelin system has also been shown to be involved in inflammation process and in the development and progression of several types of cancers (6).

Ghrelin Gene Derived Peptides

The native, intact ghrelin molecule was originally reported to be encoded by a single-copy gene called GHRL, which is located on the short arm of chromosome 3 in humans, which was initially thought to be composed of four coding exons (exons 1-4) (1). However, it is now known that the human GHRL gene contains a number of upstream first exons (exon -1, exon 0 and extended exon 1) that can act as alternative sites for transcription initiation (7). The originally identified mRNA transcript from the ghrelin gene in humans encodes a 117-aa long peptide called pre-pro-ghrelin (1). This peptide has been also identified in a number of mammalian species, where its amino acid sequence is highly conserved among the orthologous mammalian ghrelin pre-pro-peptides (8). Specifically, the human pre-pro-ghrelin contains a signal peptide and a 94-aa segment called pro-ghrelin, which undergoes proteolytic processing that leads to the generation of the mature/native ghrelin peptide and an additional C-terminal peptide named C-ghrelin. Moreover, the C-ghrelin can subsequently undergo a further proteolysis process resulting in the generation of obestatin, which was initially thought to be the antagonist hormone for ghrelin (9). Importantly, the relevance of these peptides, native-ghrelin and obestatin, across mammalian species is suggested by the fact that the sequences of both hormones are highly conserved among mammalian genes (8).

Regarding native ghrelin, it is worth mentioning that the ghrelin peptide is subjected to a unique modification, well conserved across mammalian species, consisting of the acylation of the third serine residue (10). According to the acylation status, unacylated ghrelin (UAG) can be distinguished from acylated-ghrelin, which is the initially identified peptide and corresponds to the active peptide that binds to its canonical receptor (GHSR-1a, in humans). The UAG represents over the 90% of total blood ghrelin, but despite its comparatively larger proportion in the body, the process underlying the *in vivo* production of this unacylated version of the ghrelin peptide remains unclear. The existence of UAG could be explained by an incomplete acylation of the nascent ghrelin peptide, or resulting from the deacylation of the mature acylated-ghrelin. In that UAG exerts some biological functions, but does not bind the classic ghrelin receptor, there must exist an alternative ghrelin receptor that mediates the actions of UAG. Importantly, the acylation process has recently been identified to be mediated by an enzyme called Ghrelin-O-acyl-transferase or GOAT (11, 12), however, although some initial results suggest that the acylation occurs before the final maturation of the pre-pro-ghrelin peptide, it is still not completely clear whether the acylation of the ghrelin precursor occurs before or after the protease processing (13).

A more profound study of the regulation of the ghrelin gene has shown the existence of a large number of alternative mRNA molecules generated by different

processes of alternative splicing. Some of these alternative molecules exhibit small changes in sequences, as is the case of des-Gln14-ghrelin, which is identical to native ghrelin except for the deletion of one glutamine (14). The differences in function between this 27-aa peptide hormone and the native ghrelin peptide are still to be elucidated. Another splicing variant from the ghrelin gene appears when Exon 3 is deleted. This variant, named D_3-C-ghrelin, encodes, in humans, a 91-aa peptide that lacks the region coding for obestatin (15). Although the functions of this peptide remain unknown, it has been shown to be overexpressed in human prostate and breast cancer, strongly suggesting that the peptide is functional.

Consistent with an overexpression of ghrelin gene variants in tumor samples, our group has recently identified a novel variant generated by the retention of the intron 1 and consequently named In1-ghrelin that has been found to be over-expressed in breast (16), pituitary and prostate cancers. The In1-ghrelin variant shares the signal peptide and the initial portion of the peptide with native ghrelin; however, the amino acid sequence of the In1-ghrelin is subsequently altered by the retention of the intron. Supporting a physiological role of this new variant, its orthologous counterparts has been identified in mice, called In2-ghrelin (17), and in a non-human primate model, baboons (16). Similar to that observed for the native ghrelin pre-pro-peptide, In1-ghrelin pre-pro-peptide also exhibits sites for putative protease action, suggesting that the full peptide could be processed to yield mature, functional peptides.

Ghrelin Gene Products in the Control of Mammalian Pituitary Function

Ghrelin and some of the ghrelin gene derived products are produced in a wide variety of tissues. In some cases, different ghrelin gene derived variants have been found to be co-expressed in the same tissue, suggesting that some of those variants could be playing a relevant role in these tissues. Considerable efforts have been implemented in order to separate out the roles of the ghrelin gene derived peptides on the modulation of several (patho)-physiological functions in different species. Of particular interest is the role that these old and new molecules play in the regulation of pituitary gland secretions, which are key regulators of body homeostasis. Therefore, a combination of *in vivo* and *in vitro* studies using animal (including non-human primates) models and humans have been applied.

As previously mentioned, native ghrelin was initially identified by its ability to stimulate GH secretion *in vitro* in several species such as rats, pigs and baboons (18). In particular, it was demonstrated that native ghrelin is as potent as GHRH, the classical GH-releasing factor, in the stimulation of GH secretion (19). In addition, the crucial role of native, acylated, ghrelin in the control of GH secretion has been demonstrated *in vivo*, where acylated ghrelin potently stimulated GH release in humans and rodents, and has been shown to be directly involved in the generation of the pulsatile GH secretion pattern (20). In contrast to acylated ghrelin, no effect of UAG has been found in the modulation of GH production/secretion.

In striking contrast, the role that obestatin may play in the modulation of GH secretion has classically been controversial (9, 21, 22). While some studies observed no effect of obestatin on GH secretion, other studies found a stimulatory action. In

order to shed light on this controversy, our group has analyzed the role that obestatin plays on GH secretion by using a dual, *in vivo* and *in vitro*, approach. Recent results (Luque RM et al., unpublished) have revealed that, in line with a potential opposite role originally attributed to obestatin with respect to ghrelin, obestatin treatment in male mice induces a reduction in plasma GH and in pituitary GH expression *in vivo*. In order to analyze whether obestatin plays a direct role in GH production acting at the pituitary level, *in vitro* mouse and non-human primate (baboon) pituitary cells cultures were also used. Our results also demonstrate that obestatin directly inhibits GH expression and secretion in both mammalian species.

Interestingly, and in support of a physiological role of the mouse In2-variant, treatment with In2-ghrelin peptide directly stimulated GH secretion in mouse primary pituitary cultures, suggesting that mouse In2-ghrelin variant may play a relevant role in regulating somatotrope function in mice. In addition, the human counterpart of mouse In2-ghrelin (In1-ghrelin) also seems to play a relevant role in the control of pituitary GH secretion. A preliminary study has shown that the In1-ghrelin variant is overexpressed in GH-producing tumors in comparison with normal pituitaries. In addition, treatments with In1-ghrelin derived peptides increased GH secretion in primary pituitary cultures derived from acromegaly patients (our unpublished results).

Similarly to that observed for GH, ACTH secretion has also been observed to be stimulated in response to native, acylated, ghrelin or to synthetic GH-secretagogues in humans and rats (20). However, parallel experiments indicated that UAG was not able to exert any effect in terms of modulation of ACTH production and/or secretion.

In order to study the role of obestatin on ACTH release, a similar approach was implemented by injecting obestatin into male C57Bl/6 mice. These studies demonstrated that obestatin is able to induce an increase in plasma ACTH and in expression of POMC, the precursor of ACTH, in the pituitary. To analyze whether obestatin plays a direct role in ACTH production at the pituitary level, *in vitro* mouse and baboon pituitary cells cultures were used to demonstrate that obestatin also directly stimulates ACTH expression and secretion, which suggests that obestatin, through a direct pituitary effect, could play a relevant role in regulating ACTH release in mice and primates (and probably humans).

Interestingly, the peptides derived from the In1-ghrelin and the In2-ghrelin variants exhibited similar effects to native ghrelin in the modulation of ACTH production in mouse and human pituitary samples. Specifically, the mouse In2-ghrelin variant was able to stimulate ACTH release from mouse pituitary cultures. In addition, similar to that shown in acromegaly, we found that In1-ghrelin variant was overexpressed in human ACTH-producing pituitary tumors. In addition, consistent with a patho-physiological role of In1-ghrelin, the treatment with In1-ghrelin peptides in human ACTH-producing primary cell cultures *in vitro* elicited a clear, significant increment in the secretion of ACTH (unpublished data).

Concluding Remarks

Altogether, the data gathered to date demonstrate that the ghrelin gene (GHRL) encodes for a number of different peptides generated through proteolytic process-

ing and/or alternative splicing. Importantly, the ghrelin gene structure, sequence and regulation are notably conserved among mammalian species. In addition, the ghrelin gene derived peptides exhibit a wide variety of physiological functions that can be redundant, independent or even opposite, but which, generally, are conserved across species. Therefore, a profound, comprehensive and comparative study of the ghrelin gene regulation could shed some light on the (patho)-physiological relevance of the different ghrelin gene derived peptides.

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ZEBRAFISH OVEREXPRESSING AGOUTI-SIGNALING PROTEIN 1 (ASIP1) EXHIBIT PIGMENT PATTERN ANOMALIES

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In fish, the dorso-ventral pigment polarity is achieved because a melanization inhibition factor (MIF) inhibits melanoblast differentiation and encourages iridophore proliferation in the ventrum. A previous work of our group suggested that agouti-signaling protein 1 (*asip1*) is the uncharacterized MIF. *Asip1* is mainly expressed in the ventral skin with residual expression levels in the dorsal region. High expression levels in the ventral skin are thought to inhibit melanogenesis by blocking melanocortin receptor subtype 1 (MC1R) signaling, whereas asip1 absence in the dorsal skin would allow dark pigment synthesis. To demonstrate the involvement of asip 1 in the fish pigment pattern, we generated a transgenic zebrafish line overexpressing *asip1*. We demonstrated that zebrafish overexpressing *asip1* exhibit an inhibition of melanogenic synthetic pathway in the dorsal region, but an increased iridophore proliferation that results in a brilliant stripe in the dorsal region. In conclusion, we demonstrated that the inhibition of the melanocortin system leads to pigment anomalies.

Introduction

In teleost fish, pigment cells are commonly found in the dermis and can be divided into light-absorbing (melanophores, xantophores, erythrophores and cyanophores) and light-reflecting (leucophores and iridophores) chromatophores. Fish melanophores contain eumelanins (black-brown pigments), whereas xantophores and erytrophores synthesize carotenoids and/or pteridines that contribute to the reddish and yellowish components of the skin coloration. Iridophores are commonly localized in whitish and silvery areas of the skin, predominantly on the belly surface. They contain crystalline platelets composed of purines, mainly of guanine, which are responsible for the reflection of light. Fish countershading is achieved by a patterned distribution of the pigment cells, with the light-absorbing and light-reflecting chromatophores mostly distributed in the dorsal and ventral areas, respectively (1). Experimental data in fish and amphibian species suggest that this dorso-ventral pigment pattern is achieved because a putative diffusible melanization inhibition factor (MIF), locally produced by cells in the ventral skin, inhibits melanoblast differentiation and stimulates iridophore proliferation in the ventrum (2). Our recent studies support agouti-signaling protein 1 (*asip1*) as the fish MIF (3). In goldfish, asip1 is expressed in the ventral skin, but not in the dorsal skin. It inhibits melanocortin-induced melanin dispersion in melanocytes and selectively binds melanocortin receptor 1 (MC1R, 3). Frameshift mutations introducing a premature stop codon in melanocortin MC1R or inactivating mutations in blind Mexican cave tetra (*Astyanax mexicanus*) are responsible for a decrease in the number of melanocytes and of the melanin content. This phenotype is reca-

pitulated by MC1R knockdown in zebrafish (4). Taken together, the data support that interaction between α-MSH/*asip1* and MC1R is involved in the establishment of the dorso-ventral pigment pattern, controlling chromatoblast survival, differentiation and/or proliferation as well as melanin synthesis. To assess the effect of *asip1* on dorso-ventral pigment pattern, we take advantage of zebrafish genetic and genomic tools to generate transgenic zebrafish lines overexpressing goldfish *asip1* under the control of a constitutive promoter. We demonstrate that disruption of the endogenous *asip1* expression gradient results in dorsal skin paling, due to reduction of melanophore numbers and decreased expression of genes encoding key enzymes of the melanogenic pathway in zebrafish.

Materials and Methods

The transgenic *asip1* line was generated with the Tol2 transposon system. Asip1 was subcloned into pT2AL200R1506. The Tol2-transposon-based vector pT2AL-200R150G contains the *egfp* gene under the control of elongation factor 1α (EF1α), a constitutive promoter. We replaced the *egfp* gene with *asip1* gene. A total of 250 pg of construct and synthetic 5' capped mRNA (150 pg) encoding a transposase were co-injected into WT embryos at the one- or two-cell stage, with 1% of phenol red as tracer. The pigment pattern of adult mosaic Tg(ef1alfa:asip1) fish (F0) showed a pronounced phenotype, with paler head and body than WT fish (data not shown). To obtain the F1 generation *Tg(ef1alfa:asip1)*, putative founder fish were out-crossed to WT fish, and their progeny were analyzed by *in sit*u hybridization with the antisense RNA probe for *asip*1 from goldfish. Positively identified F1 siblings were mated. The F2 embryos were grown to sexual maturity and individuals F2 were mated to wild-type fish. The F3 progeny were analyzed for the transgene. Identified homozygous F2 were mated to each other to produce a large stable homozygous (F3) population.

Results and Discussion

We generated transgenic zebrafish lines that overexpress goldfish asip1 using the constitutive ef1alfa promoter. These fish showed a distinct pigment pattern phenotype as adults (Fig. 1). Compared with WT siblings, the transgenics had reduced pigmentation over the entire dorsal region, including the head (Fig. 2). This dramatic hypomelanization was also prominent in the dorsal body stripes (Fig. 1). Dorsally, instead of the prominent covering of scale melanophores, the transgenics show a new bright band of iridophores that runs lightly dorsal to the dorsalmost stripe. Nevertheless, other aspects of the pigment pattern are unchanged, with fin pigment pattern and opercular iridophore pattern occurring in a manner indistinguishable from WT fish. The number of skin melanophores in transgenic fish was decreased by 61% in the head, 66% in the dorsum and compared to the control group ($p<0.001$). We found no differences in the melanophore number in the belly. Thus, the increase in asip1 expression reduces the number of melanophores only in the dorsal region of adult transgenic fish. By overexpressing asip1, we were able to separate two different pigment patterns in zebrafish, i.e. the dorso-ventral pigment pattern and the stripped pattern. Both patterns are differentially regulated since asip1 overexpression profoundly affects dorso-ventral pigment pattern but have modest effect on stripped pattern.

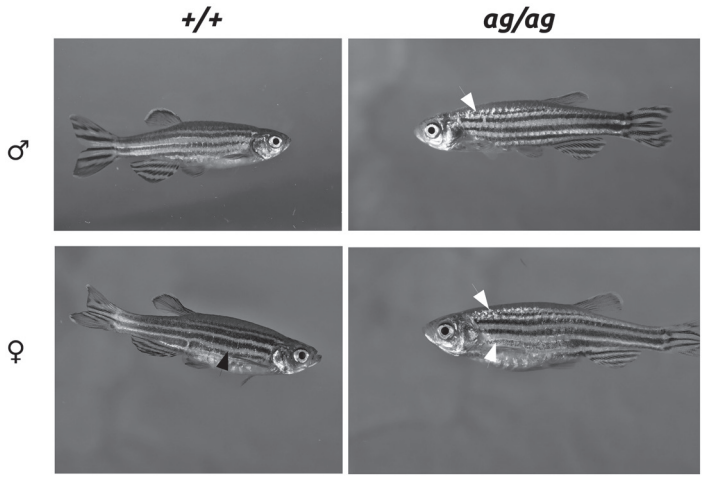

Figure 1. Lateral view of male (upper panels) and female (lower panels) wild-type, (+/+) (left panels), and transgenic, (*ag/ag*) (right panels), zebrafish overexpressing asip1 under the control of a constitutive promoter elongation factor 1α (EF1α). White arrows in the dorsal area indicate the prominent iridophore band in transgenic zebrafish that is absent in wild-type zebrafish (black arrows in the dorsal area). White and black arrows in the ventral area indicate the absence/presence, respectively of the end ventral band of the stripped pattern in females.

Figure 2. Dorsal view of wild-type (+/+) and transgenic (*ag/ag*) females overexpressing asip1. See Figure 1 for more details.

Acknowledgements

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DIFFERENTIAL GENE EXPRESSION IN RAINBOW TROUT PREADIPOCYTE CULTURED CELLS ASSOCIATED WITH PROLIFERATION AND ADIPOCYTE MATURATION

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Excessive accumulation of adipose tissue in cultured fish is an outstanding problem in aquaculture that may negatively affect animal health and productivity. To understand the development of adiposity, it is crucial to identify the factors that regulate the formation of adipocytes from precursor cells. Therefore, the transcriptomic profile at different time points (days 3, 8, 15 and 21) during primary culture of rainbow trout preadipocytes has been investigated using Agilent trout oligo microarrays. Overall, our study demonstrates the coordinated expression of functionally related genes during proliferation and differentiation of rainbow trout adipocyte cells. Furthermore, the information generated will allow the investigation of specific genes involved in particular processes of fish adipogenesis.

Introduction

The ongoing intensification of the aquaculture sector has led to the development of energetic diets with high lipid content, and to the need of finding new raw materials, most of them of vegetable origin (1). This, together with the low swimming activity that cultured fish are facing, can induce several negative consequences such as increase in fat depots, change in the fatty acid composition of the fillet, increase in oxidative stress, or early sexual maturation among others. All these consequences represent a problem for the productivity of the sector, since all this extra fat accumulated is pure loss for aquaculture.

Even though excessive accumulation of fat in both fillet and around the viscera is an unresolved problem, very little is known about the factors regulating the development and functions of adipose tissue in fish, and how an increase in fat deposition may lead to health problems. While in mammals an excess of adipose tissue, as occurs in obesity, is associated with chronic low-grade inflammation (2), which may contribute to the development of many aspects of the metabolic syndrome, it is still unknown if this is the case in fish. Increased lipid accumulation might impose considerable stress on adipocytes, leading to adipocyte activation and thus, production and secretion of inflammatory mediators.

Adipose tissue growth is achieved by hypertrophy of adipocytes as well as by proliferation and differentiation of new cells. Until recently, adipose tissue has been considered to be static and a mere storage compartment of triglycerides, but it is now clear that adipocytes are highly responsive to extracellular stimuli, play a central role in overall energy homeostasis and are also essential for certain aspects of the immune system producing numerous regulatory factors known as

adipokines (3). In order to increase the understanding of the development and functions of adipose tissue in rainbow trout, and to identify novel proteins that could serve as potential links between adipocyte growth and whole body energy homeostasis, the transcriptomic profile along adipocyte culture at four different time points has been analyzed.

Materials and Methods

1. Cell culture procedure. Cells were isolated from a pool of white adipose tissue (6-7 fish per experiment), cultured and differentiated *in vitro* as previously described (4). Four independent cultures were performed and samples were taken at day 3 (developing preadipocytes), 8 (24 h the induction of differentiation), 15 (early differentiated adipocytes) and 21 (fully differentiated adipocytes).

2. Microarray analysis. The microarray was performed using an Agilent-based platform with 8 × 60 K probes per slide (GPL15840). RNA labeling and hybridization as well as data acquisition and analysis was performed as previously reported (5).

3. Real-time PCR and western blot analysis. Total RNA from cells was extracted using the TriReagent method according to the manufacturer's instructions. cDNA synthesis and PCR assays were performed as described elsewhere (6). The protein expression of selected molecules during culture development was also analyzed by western blot analysis (4).

Results and Discussion

Our analysis identified 4026 genes differentially expressed (fold-change >3) that were divided into two major clusters corresponding to the main phases observed during the preadipocyte culture: proliferation and differentiation. Proliferation cluster comprised 1685 genes up-regulated from days 3 to 8 of culture, while the differentiation cluster was characterized by 2341 induced genes from days 15 to 21. Proliferation was characterized by the presence of genes involved in basic cellular and metabolic processes such as transcription, ribosome biogenesis, translation and protein folding. Terminal differentiation phase was enriched with genes critical for carbohydrate and lipid metabolism and adipokines. The main biological functions of differentially expressed genes associated with these two phases are presented in Figure 1.

One of the first hallmarks of adipogenesis is the dramatic alteration in cell shape, since preadipocytes are transformed from a fibroblast to a spherical shape. Our analysis suggested that the balance between members of the matrix metalloproteinase family and their inhibitors might play a prominent role during both early and late phases of adipocyte development by regulating cell shape and extracellular matrix remodeling. In mammals, cell shape and extracellular matrix remodeling have been found to regulate preadipocyte commitment and competency by modulating among others, the RHO-family GTPase signaling cascade (7). This seems to be the case for rainbow trout adipocytes, since our data revealed the presence of a large number of genes from this family up-regulated during both phases of adipogenesis; proliferation and differentiation.

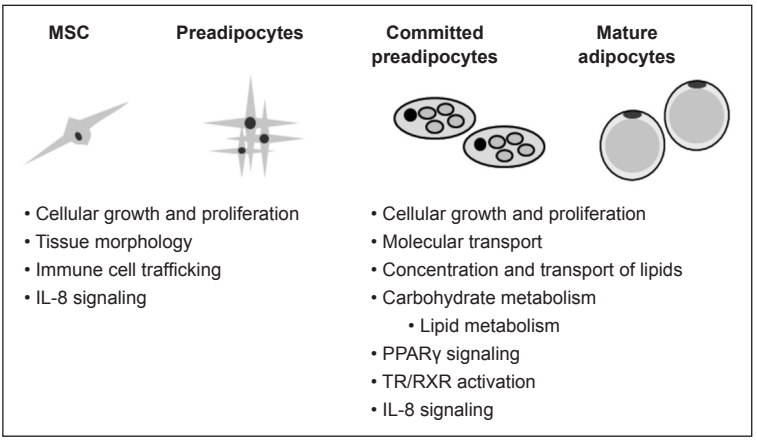

Figure 1. Functions significantly enriched during the phases of proliferation and differentiation of rainbow trout preadipocytes in culture.

All this morphological modification has been shown to regulate adipogenesis also by promoting the expression of critical transcription factors (8). The activation of these transcription factors is required in the adipocyte maturation process since they are responsible for the coordinated induction and silencing of a large number of genes related to the regulation of adipocyte morphology and functions. In addition to CCAAT/enhancer-binding proteins, a large set of transcription factors were identified, including the early growth response 2 (Krox20) and GATA2. Several members from the Krüppel-like factor family were identified exhibiting an expression pattern during adipocyte development, which indicates that a cascade of these factors might be working during fish adipogenesis highlighting therefore their implication in fish fat biology. Furthermore, microarray results suggested the existence of an important link between transcriptional regulation and epigenetic modulation, due to the large number of expressed genes encoding epigenetic transcriptional regulators, especially present during the proliferation phase.

The development of the immune functions was revealed early from the first steps of adipocyte differentiation. The severe architectural modifications faced by these cells during their life cycle in order to become a fully functional adipocyte, controlling the energy balance by storing and mobilizing triglycerides and paying a pivotal role as an endocrine and paracrine cell, might trigger the expression of many immune-related genes. The proinflammatory cytokine tumor necrosis factor (TNFα), as well as a panel of TNFα-related genes and receptors, was upregulated before cell confluence. In terrestrial vertebrates, TNFα is known to be a potent negative regulator of adipocyte differentiation (9), and this has been confirmed in fish too (4). Hence the fact that the TNFα-axis exhibited a down regulation during the last days of culture might be an important signal for the onset of adipocyte differentiation. During the last phase of adipocyte maturation many proteins involved in metabollic trapping, glycogen synthesis, the pentose phosphate pathway, glycolisis and essentially all aspects of lipogenesis were expressed. Some of

the metabolic and structural adipocyte-related molecules were analyzed at mRNA and protein level (Figure 2) to validate the microarray analysis; and the results confirmed the mature state of the cells.

All in all, the microarray analysis revealed that genes over-expressed during rainbow trout primary cultured adipocyte development can be divided into two different groups with different temporal profiles, characterizing the two main stages of adipogenesis: proliferation and differentiation. All the information generated provides a basis for understanding the physiological mechanism that underlies adipogenesis, and could yield insights into particular cellular and molecular events involved in this intricate process.

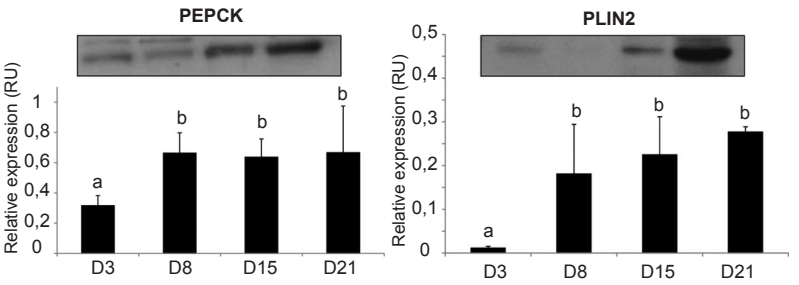

Figure 2. Phosphoenolpyruvate carboxylase (PEPCK) and perilipin 2 (PLIN2) mRNA (real-time PCR) and protein levels (western blot) during the development of rainbow trout preadipocytes in culture. Values are expressed as mean ± S.E.M. (n=4). Values not sharing letters are significantly different ($P<0.05$, ANOVA followed by Tukey's test).

Acknowledgements

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EFFECTS OF OLEATE OR OCTANOATE INTRACEREBROVENTRICULAR TREATMENT ON FOOD INTAKE AND HYPOTHALAMIC FATTY ACID SENSING SYSTEMS IN RAINBOW TROUT

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Most teleosts are relatively intolerant to glucose, thus relying more on amino acid and lipid metabolism for fueling purposes. Reduced food intake has been observed in fish fed with lipid-enriched diets or containing high fat stores suggesting the presence of lipid sensing systems. Previously, we demonstrated that acute administration of oleate (long-chain fatty acid, LCFA) or octanoate (medium-chain fatty acid, MCFA) either *in vivo* (intraperitoneal) or *in vitro* (in the absence of external influences) induced a response in the hypothalamus which is compatible with fatty acid (FA) sensing system in parallel with decreased food intake. If FAs are directly sensed in the hypothalamus, central administration should elicit effects similar to those of IP treatment, as described in mammals. Accordingly, in the present study, we observed after intracerebroventricular (ICV) administration of oleate or octanoate in rainbow trout effects similar to those of IP treatment, such as reduced potential of lipogenesis and FA oxidation and decreased potential of ATP-dependent inward rectifier potassium channel (K^+_{ATP}), as well as food intake. Those responses are compatible with fatty acid sensing in which fatty acid metabolism, binding to FAT/CD36 and mitochondrial activity appear to be involved. Changes in those hypothalamic pathways can also be related to the control of food intake, since changes in mRNA abundance for some specific neuropeptides such as NPY and POMC were also noted.

Introduction

Specialized neurons within the mammalian hypothalamus have been suggested to detect changes in plasma levels of long-chain fatty acid (LCFA), but not short-chain (SCFA) or medium-chain (MCFA) fatty acids (FA). Moreover, enhanced lipid levels are known to inhibit food intake in mammals. In fish, a previous study in rainbow trout (*Oncorhynchus mykiss*) (1) demonstrated that intraperitoneal (IP) acute administration of oleate (LCFA) or octanoate (MCFA) elicited an inhibition in food intake and induced in the hypothalamus a response compatible with FA sensing in which FA metabolism, binding to FAT/CD36 and mitochondrial activity were apparently involved. Changes in those hypothalamic pathways can be also related to the control of food intake, since changes in mRNA levels of specific neuropeptides such as NPY and POMC were also noted (1). In a subsequent study (2) we observed that the rainbow trout hypothalamus *in vitro* displayed responses in parameters related to FA-sensing similar to those previously observed after IP treatment allowing us to suggest that the increase of circulating LCFA or MCFA levels in rainbow trout is directly sensed in the hypothalamus. However, we cannot discard the possibility that the effects of intraperitoneal (IP) treatment with FA could be attrib-

uted to an indirect effect mediated by changes elicited in levels of peripheral hormones. Thus, we have evaluated in rainbow trout the effects of intracerebroventricular (ICV) treatment with oleate or octanoate on food intake and in parameters related to putative FA sensing systems in the hypothalamus. If FAs are directly sensed in the hypothalamus, central administration should elicit effects similar to those of intraperitoneal treatment.

Materials and Methods

Rainbow trout of about 80 g body mass were obtained from a local fish farm (A Estrada, Spain) and were maintained for 1 month in 100 liter tanks under laboratory conditions and a natural photoperiod in dechlorinated tap water at 15 °C. On the day of the experiment, fish were lightly anesthetized with MS-222 (50 $mg \cdot l^{-1}$) buffered to pH 7.4 with sodium bicarbonate, and weighed. ICV was carried out by dispensing $1\mu l \cdot 100g^{-1}$ body mass of Hanks´ saline alone or containing 1 μmol oleate or octanoate. In a first set of experiments, food intake was registered for 3 days before treatment (to define basal line data) and then 6 and 24 h after ICV treatment with saline alone (control, n=10) or containing oleate (n=10) or octanoate (n=10). In a second set of experiments, fish were ICV injected with saline alone (control, n=30) or containing oleate (n=30) or octanoate (n=30) with the same concentrations described above. After 2 h or 6 h, fish were lightly anesthetized with MS-222 (50 $mg \cdot l^{-1}$) buffered to pH 7.4 with sodium bicarbonate. Blood was collected to assess metabolite levels and 3 different sets of hypothalamus pools were used: to assess enzyme activities, tissue metabolites, and mRNA levels. Parameters were assessed as previously described (3, 4, 5).

Results and Discussion

Changes in food intake are shown in Fig. 1A. Oleate or octanoate treatment decreased food intake after 6 h and 24 h compared with controls, and the decrease was lower after 24 h for both FAs.

Levels of plasma metabolites were not significantly different in any parameter assessed due to treatment with oleate or octanoate (data not shown).

In the hypothalamus, fatty acid levels (Fig. 1B) increased compared with controls after treatment with oleate (2 h) and octanoate (2 and 6 h), and levels after octanoate treatment were higher at 6 h than at 2 h. Enzyme activities in the hypothalamus are shown in Fig. 2. Compared with controls, FAS activity (Fig. 2A) decreased after 2 h treatment with oleate or octanoate whereas activity increased after 6 h treatment with octanoate; activities after 6 h treatment with oleate or octanoate were higher than those after 2 h. CPT-1 activity (Fig. 2B) decreased after 2 h and 6 h treatment with oleate or octanoate, and the activity was lower after octanoate treatment.

Changes in mRNA levels assessed in the hypothalamus are shown in table 1. Oleate treatment compared with controls down-regulated mRNA levels of FAT/CD36 (2 h and 6 h), ACC (2 h), ACLY (2 h), CS (2 h), FAS (2 h), Kir6.x-like (2 h and 6 h), SUR-like (2 h and 6 h), LXRα (2 h and 6 h), and NPY (2 h), and up-regulated those of CS (6 h), MCD (2 h), CART (2 h), and POMC (2 h and 6 h). The treatment with

octanoate compared with controls resulted in down-regulation of mRNA levels of ACC (2 h), Kir6.x-like (2 h), SUR-like (2 h and 6 h), and NPY (2 h), and up-regulation of mRNA levels of CPT-1c (2 h), CS (6 h), MCD (2 h), UCP2a (2 h), and POMC (2 h and 6 h). Changes elicited after 2 h treatment with octanoate were significantly different than those elicited at the same time by oleate for mRNA levels of ACLY, CPT-1c, FAS, UCP2a, and LXRα; no such differences were noted after 6 h. Changes induced by 6h treatment with oleate were significantly different than those observed after 2 h treatment with the same FA for ACC, ACLY, FAS, CART, and NPY. Changes induced by 6 h treatment with octanoate were significantly different than those observed after 2 h treatment with the same FA for ACLY, CPT-1c, UCP2a, and NPY.

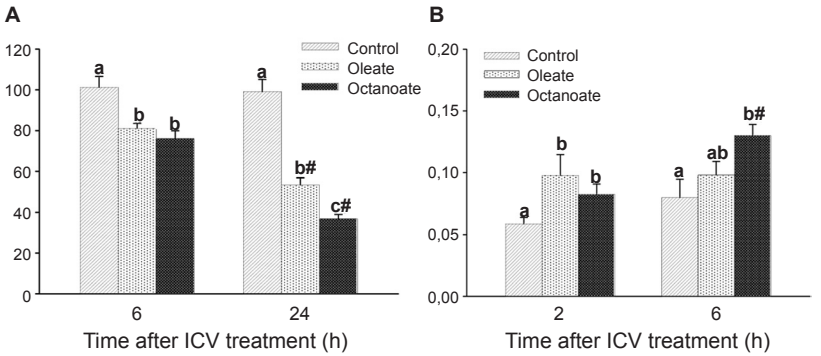

Figure 1. A) Food intake (% basal levels) in rainbow trout 6 and 24 h after intracerebroventicular administration of 1 μl saline alone (control) or containing 1 μmol oleate or octanoate. Different letters indicate significant differences (P<0.05) among treatments within each time. #, significantly different (P<0.05) from 6 h at the same treatment. The results are shown as mean + S.E.M. of the results obtained in the three different tanks in which 10 fish were used per group in each tank. B) Levels of FA (μmol/g) in the hypothalamus of rainbow trout after 2 h or 6 h of intracerebroventicular administration of 1 μl saline alone (control) or containing 1 μmol oleate or octanoate. Each value is the mean + SEM of n = 10 fish per treatment. Different letters indicate significant differences (P<0.05) among treatments within each time. #, significantly different (P<0.05) from 2 h at the same treatment.

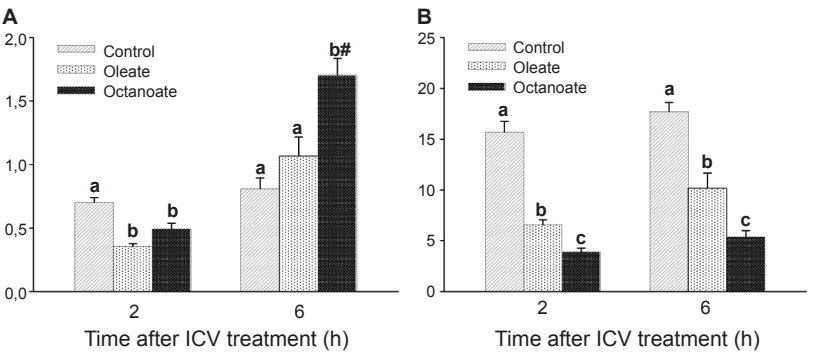

Figure 2. Activities(mU/mg protein) of FAS (A) and CPT-1 (B) in the hypothalamus of rainbow trout after 2 h or 6 h of intracerebroventicular administration of 1 μl saline alone (control) or containing 1 μmol oleate or octanoate. Each value is the mean + SEM of n = 10 fish per treatment. Different letters indicate significant differences (P<0.05) between treatments within each time. #, significantly different (P<0.05) from 2 h at the same treatment.

Table 1. mRNA levels in the hypothalamus of rainbow trout after 2 or 6 h of intracerebroventicular administration of 1 *µ*l saline alone (control) or containing 1 *µ*mol oleate or octanoate.

	Oleate		Octanoate	
	2 h	**6 h**	**2 h**	**6 h**
ACC	-1.54*	+1.15#	-1.38*	+1.09
ACLY	-1.46*	+1.07#	+1.26†	-1.05#
CART	+1.38*	-1.18#	+1.03	-1.09
CPT-1c	-1.04	+1.23	+1.63*†	+1.09#
CS	-1.41*	+1.43*	+1.13	+1.49*
FAS	-1.40*	+1.20#	+1.19†	-1.07
FAT/CD36	-1.37*	-1.51*	+1.09	-1.24
Kir6.x-like	-1.43*	-1.34*	-1.36*	-1.18
LXRα	-1.68*	-1.34*	+1.11†	+1.03
MCD	+1.44*	+1.13	+1.80*	+1.21
NPY	-1.51*	+1.01#	-1.53*	-1.03#
POMC	+1.69*	+1.71*	+1.55*	+1.58*
SUR-like	-1.35*	-1.54*	-1.34*	-1.69*
UCP2a	+1.27	+1.04	+2.80*†	+1.23#

Data is expressed as fold-induction (+, increase; -, decrease) with respect to the control group. *, significantly different (P<0.05) from control fish at the same time and treatment. #, significantly different (P<0.05) from 2 h at the same treatment. †, significantly different (P<0.05) from oleate treatment at the same time.

The results obtained support direct FA sensing of the rainbow trout hypothalamus to increased levels of oleate and octanoate through reduced potential of lipogenesis and FA oxidation, and decreased potential of K^+_{ATP}. The FA sensing through binding to FAT/CD36 and subsequent expression of transcription factors also appears to be direct, but an interaction induced by changes in levels of peripheral hormones cannot be discarded. The activation of those systems would be integrated in the hypothalamus resulting in altered production of anorexigenic and/or orexigenic factors explaining the reduced food intake observed after IP and ICV treatment with oleate or octanoate.

Acknowledgements

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EFFECTS OF NUTRITIONAL STATUS ON PLASMA LEPTIN LEVELS AND IN VITRO REGULATION OF ADIPOCYTE LEPTIN EXPRESSION AND SECRETION IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

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The aim of the study was to explore the modulatory effect of adiposity on plasma leptin, as well as the regulatory role of hormones and nutrients on the expression and secretion of leptin from adipocytes in rainbow trout. Fish were fed a high-energy (HE) diet at two different ration levels, *ad libitum* (AL group) or 25% of satiation (RE group) for eight weeks. RE fish had significantly reduced growth and adipose tissue weight in comparison to AL fish, but increased plasma leptin levels (p=0.022). Interestingly, and contrary to mammals, plasma leptin was negatively correlated with adipose tissue mass and mesenteric fat index, suggesting that plasma leptin in fish can still convey information on the energy status of the body. Next, isolated adipocytes from fish on both ration levels and fish fed a regular diet were used to study *in vitro* the factors that may regulate leptin expression and secretion. Adipocytes were treated with insulin, ghrelin, leucine or eicosapentaenoic acid (EPA) or left untreated (control). In regular-diet adipocytes, both insulin and ghrelin increased leptin secretion dose-dependently (p=0.002 and p=0.033, respectively), and insulin showed a tendency to reduce leptin expression. Results from the HE diet fish supported the findings on plasma leptin levels, with significantly higher leptin secretion in control adipocytes from RE compared to AL fish (p=0.022). Furthermore, no significant effects on treatment were observed, neither in leptin expression nor secretion, for the AL group. In RE fish, leptin expression remained unchanged, whereas leptin secretion was significantly reduced by leucine (p=0.025), and had a tendency to increase after insulin treatment and to decrease with EPA. These data indicate that regulation of leptin secretion from adipocytes occurs mainly at a post-transcriptional level in rainbow trout and is modulated by the nutritional history of the fish.

Introduction

In mammals, leptin is primarily secreted by adipose tissue, the plasma leptin levels are known to correlate with adipose tissue mass, and it is considered that leptin works as an adiposity signal (1). Leptin secretion from adipocytes is stimulated by anabolic signals (i.e. food consumption, insulin, ghrelin, leucine and EPA), whereas it is inhibited by catabolic signals (i.e. fasting or growth hormone) (2). In the present study, we have explored in rainbow trout a) if plasma leptin acts also as an adiposity signal and b) the possible nutritional and/or hormonal regulators of leptin expression and secretion in isolated adipocytes.

Materials and Methods

Adult rainbow trout were fed *ad libitum* (AL) (n=44) or with a 25% of that ration (Restricted; RE) (n=42) with a commercial high-energy (HE) diet for eight weeks to obtain fish with two different levels of adiposity. Biometrics and plasma samples were obtained at time 0 (t=0) and at the end of the trial (t=8) to analyze plasma leptin by radioimmunoassay (RIA) and metabolites (glucose, triglycerides; TG and free fatty acids; FFA) using commercial kits. Visceral adipose tissue from fish at both ration levels, as well from regular diet fish, was excised and adipocytes were isolated as described by Albalat et al. (3). Around 2 million cells were incubated for 3 h with; a) different doses of insulin (I) (10 and 100 nM), ghrelin (G) (0.1, 1 and 10 nM) or left untreated (control; C) for the regular diet adipocytes (n=5-6) and, b) Insulin (Ins) 100 nM, ghrelin (Ghrel) 10 nM, leucine (Leu) 5mM or eicosapentaenoic acid (EPA) 100 μM or left untreated (control; Ctrl) (n=6-7 with 2-4 replicates). After incubation, the medium was used to analyze leptin secretion by RIA, and the cells to analyze by real time quantitative PCR leptin A1 (LepA1) mRNA expression.

Results and Discussion

1. Biometrics. AL fish had significantly increased growth and adipose tissue weight in comparison to RE fish after eight weeks of feeding on the HE diet (-47% and -52%, respectively; $p<0.01$); an also in comparison with t=0, but adipose tissue weight of RE fish did not change with time. These results corroborate the fact that the experimental design used was effective in generating two groups of fish with clearly differentiated nutritional status and fat reserves.

2. Plasma parameters. At t=8, plasma glucose remained unchanged, TG increased in both groups, but FFA increased in AL fish only (data not shown). Plasma leptin levels were higher in RE fish in comparison with AL fish ($p=0.022$) (Fig. 1A). This supports the hypothesis by Fuentes et al. (4), who suggested that increased leptin levels may be linked to a survival behavior in fish, which experience naturally prolonged periods of food shortage, lowering appetite and limiting energy-wasting foraging activity.

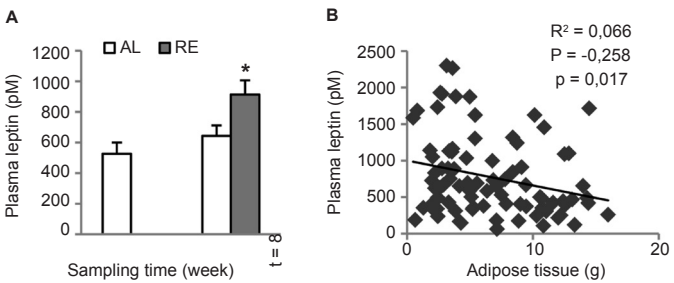

Figure 1. (A) Plasma leptin levels in *ad libitum* (AL) and feed restricted (RE) rainbow trout. Data at different sampling times (week t=0 and t=8) are shown as mean ± SEM. (n=8 for t=0 and n=42-43 for t=8). No differences were observed between sampling times within the AL or RE group. Asterisk indicates significant differences between groups at t=8 ($p<0.05$). (B) Correlation between plasma leptin and adipose tissue weight of AL and RE rainbow trout at week t=8. R2 of the linear regression and correlation coefficient (P) and p value (p) from the Pearson correlation are indicated.

Moreover, contrary to the case of mammals, plasma leptin in rainbow trout was significantly negatively correlated with adipose tissue mass (Fig. 1B), mesenteric fat index and liver weight (data not shown), indicating that plasma leptin might act as an endocrine signal of adiposity in rainbow trout.

3. Leptin expression and secretion in isolated adipocytes. Leptin secretion from isolated adipocytes of fish fed a regular diet increased dose-dependently after incubation with insulin or ghrelin (p=0.002 and p=0.033, respectively) (Fig. 2A and 2B). However, LepA1 expression did not change upon treatment (data not shown), thus suggesting that regulation of leptin secretion from adipocytes in rainbow trout occurs mainly at a post-transcriptional level.

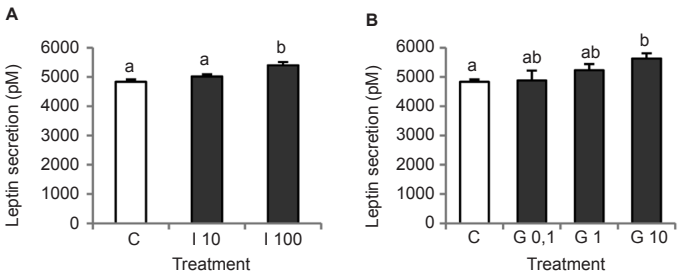

Figure 2. Leptin secretion into the medium in isolated adipocytes from rainbow trout fed a regular diet after (A) insulin or (B) ghrelin treatment. Adipocytes were left untreated (C) or treated either with insulin at 10 or 100 nM (I10 or I100) or ghrelin at 0,1, 1 or 10 nM (G0,1, G1 or G10) for 3 h. Data are shown as mean ± SEM (n=5-6). Different letters indicate significant differences between treatments (p<0.05).

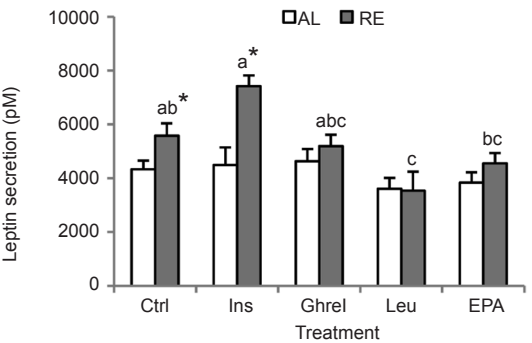

Figure 3. Leptin secretion in *ad libitum* (AL) and feed restricted (RE) rainbow trout isolated adipocytes. Adipocytes were left untreated (Ctrl) or treated either with insulin 100 nM (Ins), ghrelin 10 nM (Ghrel), leucine 5 mM (Leu) or EPA 100 μM (EPA) for 3 h. Data are shown as mean ± SEM (n=6-7 with 2-4 treatment replicates per group). Different capital or lower case letters indicate significant differences between treatments within the AL or RE group, respectively, and asterisks indicate significant differences between groups within treatments (p<0.05).

Leptin secretion from the adipocytes of the HE diet fish supported the findings on circulating plasma leptin, with significantly higher leptin levels in the media of

control adipocytes from RE than AL fish (p=0.022), as well as in the media of insulin-treated adipocytes from RE fish (p=0.005) (Fig. 3). Thus, indicating that RE adipocytes retained some kind of metabolic memory. In AL fish, leptin secretion was not modified after any treatment, suggesting they might have become unresponsive. In RE fish, adipocytes incubated with anabolic hormones such as insulin, showed an increase in leptin secretion, in line with the results observed in regular diet adipocytes. Furthermore, leptin secretion in RE adipocytes was significantly reduced by leucine in comparison with the control treatment (p=0.025) (Fig. 3) and a similar trend was observed with EPA. We might speculate that these nutrient increments could be signaled by the fish as food availability, causing a change in the metabolism by decreasing leptin secretion, which in turn induces the animal towards a feeding behavior. On the other hand, LepA1 expression did not change in response to any treatment, neither in AL nor RE isolated adipocytes (data not shown); thus supporting a post-transcriptional regulation.

Altogether, our findings suggest that the physiological role of leptin is linked to the degree of adiposity in rainbow trout and support the previous hypothesis about leptin regulating food intake and energy expenditure. Furthermore, leptin seems to be a player under the influence of other important metabolic/appetite regulating hormones such as insulin and ghrelin, which modulate leptin secretion; overall suggesting that interactions between these three hormones are crucial for the regulation of metabolism and energy balance. Also, our data reveal several aspects of the underlying mechanisms regulating leptin secretion from adipocytes in this species, which can be related to the nutritional status of the fish. Finally, the present work shows for the first time a negative correlation between plasma leptin and adipose tissue mass in rainbow trout and provides further evidence that trout fed *ad libitum* with HE diets produce excessive fat accumulation that may eventually affect the animals' health and the quality of the aquaculture product.

Acknowledgements

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PROPYLTHIOURACIL TREATMENT AND SALINITY TRANSFER ALTERED 5´-DEIODINATION ACTIVITY IN GILTHEAD SEA BREAM (*SPARUS AURATA*)

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Outer ring deiodination (ORD) activity is tightly regulated by iodothyronines in mammals. In fish, discrete features of deiodinases make the whole picture much more complicated and, as a result, the regulation of ORD activity after alterations in the thyroidal status of the animal seems to be distinctive when comparing different species. In this study, we altered the thyroidal status of seawater-acclimated gilthead sea bream specimens inhibiting thyroid hormone secretion by PTU treatment for 36 days, and after that we abruptly submitted specimens for 8 days to different environmental salinities (5, 40 and 55 ppt), an environmental factor known to alter the thyroidal status in this species. Specimens treated with PTU showed a better growth; however plasma metabolite concentrations did not significantly differ between groups. This indicates that both groups, which represent two different thyroidal conditions, can reorganize their energy metabolism. Gill Na/K-ATPase (NKA) activity seems to be higher in the PTU group when submitted to hyperosmotic environments. Gill and kidney ORD activity, when measured by incubation of those tissues with T4, increased its values after 8 days of acclimation to different environmental salinities in PTU-groups. Our results show that ORD in osmoregulatory organs is sensitive to the hypo-thyroidal status induced by PTU. However, no apparent relation between ORD activities and enzyme NKA was found. This suggests that the regulation of NKA activity in gilthead sea bream does not follow the same pattern as in mammals, where changes in the thyroidal status affect this enzyme.

Introduction

The thyroid system is constituted by many elements such as thyroid hormones (THs), deiodination enzymes, carrier proteins and membrane transporters among others (1). Triiodothyronine (T3) is the biologically active hormone in fish, but the thyroid follicles secrete mainly thyroxine (T4) which is converted to T3 by two enzymes, D1 and D2, with outer-ring deiodination (ORD) activity (2). THs play many roles in animal metabolism, reproduction, metamorphosis or even salinity acclimation (3, 4). At different salinity environments, gilthead sea bream (*Sparus aurata*) modifies its thyroid system presenting a hyperthyroid state in hyposmotic environments while hypothyroidism is shown at high salinity conditions (5). It is known that hyperthyroidism suppresses D1 and D2 activities and mRNA expressions, while hypothyroidism increases them in fish (2). These changes in the thyroid system are thought to modify the metabolic pathways and thus, many *in vivo* studies have been focused on the actions exerted by anti-thyroid drugs such as

6-N-propyl-2-thiouracil (PTU), with different results depending on the species or ways of administering the drug (6, 7). For this reason, we studied the effects of PTU on the thyroid system of *S. aurata* when submitted to different salinity conditions.

Material and Methods

Juveniles of gilthead sea bream (*S. aurata*) (n=160, 13.6 ± 0.1 cm length and 43.3 ± 0.6 g weight) were maintained in four truncated-cone 1000 L tanks at 40 ppt, 19 ºC and natural photoperiod (spring in the south of Spain). Two of them were fed with a control diet while the others were fed with the same diet supplemented with 2-propylthiouracil (Sigma, P-3755) in a dose of 5 mg PTU (Kg fish^{-1} day^{-1}) as described elsewhere (7). After 36 days, 8 animals per group were sampled and the others were submitted to different environmental salinities (5, 40 and 55 ppt). After 8 days, plasma, gills and kidney samples were collected. Fork length, wet weight, plasma metabolites, and gill and kidney Na/K-ATPase as well as T4-ORD activities were measured (4).

Results and Discussion

Gilthead sea bream juveniles fed with a PTU complement exhibit higher growth than the control group, although both groups do not show significant differences in plasma metabolites, indicating an energy reallocation (Table 1).

Table 1. Effects of PTU after 36 days of treatment in *S. aurata*. (Mean±SEM, n=8). * indicate differences between groups (Student-T test, p<0.05).

Group	Length (cm)	Weight (g)	Gill Na/K-ATPase act.	Plasma lactate (mM)	Plasma free amino acids (mM)
Control	16.0±0.1	77.0±1.7	11.5±0.9	0.9±0.1	19.3±1.2
PTU	16.6±0.2*	88.2±3.6*	15.0±0.9*	0.7±.0*	13.7±1.6*

Gills and kidney are considered the most important osmoregulatory tissues in fish and NKA activity is usually studied as a key role enzyme in this process (4). After 36 days of treatment, there were no differences between the kidney NKA activities, or in the gills and kidney T4-ORD activities of both groups (data not shown). This could indicate that gilthead sea bream fed with PTU have an increased metabolism which makes the animals grow more with few changes in their plasma metabolites. This could increase the aerobic respiration and therefore, gill NKA activity.

As PTU affects the metamorphic pathways and presents inhibitory effects on larval development (6) and decreased THs levels (8), its metabolic effects will depend on the developmental stage of the fish. PTU-induced hypothyroidism does not affect NKA activity in freshwater tilapia (8), while the present study demonstrates that PTU increases not only this enzymatic activity in gills or kidney, but also T4-ORD activity in both tissues in gilthead sea bream (Table 2).

Nevertheless, the relationships between those two enzymatic activities are not clear within the present study, suggesting different pathways of regulation in fish

and mammals. In the latter, changes in the thyroidal status affect this enzyme. Further studies will be necessary to fully understand this process, such as mRNA expression of the different counterparts or plasmatic levels of THs or cortisol, another osmoregulatory key hormone.

Table 2. Effects of PTU and environmental salinity on Na/K-ATPase (µmol ADP mg prot^{-1}h^{-1}) and T4-ORD (fmol µg^{-1} min^{-1}) activities in *S. aurata*. (Mean±SEM, n=8). Different letters indicate statistical differences among groups (Two way ANOVA followed by Tukey´s posthoc test, p<0.05).

Group	**Control**			**PTU**		
Salinity (ppt)	**5**	**40**	**55**	**5**	**40**	**55**
Gill NKA	8.1±0.9 E	13.3±1.4 CD	19.7±1.4 BC	9.7±1.1 DE	23.9±1.2 AB	33.1±4.3 A
Kidney NKA	16.6±1.5 A	9.3±0.5 B	14.5±1.1 AB	13.2±1.5 AB	15.2±1.2 A	15.1±1.3 A
Gill T4-ORD	212±42 BC	100±20 C	243±35 ABC	364±35 A	293±30 AB	291±31 AB
Kidney T4-ORD	195±42 C	235±39 BC	219±29 BC	322±42 AB	408±75 AB	481±66 A

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REGULATION OF INTERMEDIARY METABOLISM BY THYROID AND INTERRENAL SYSTEMS IN THE GILTHEAD SEA BREAM (*SPARUS AURATA*)

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Thyroid and interrenal systems are involved in energy metabolism. Both endocrine pathways produce hormones (T3 and cortisol, respectively) which are considered hyperglycemic, however their effects in fish are still not clear. The aim of this study was to elucidate the relationships of these two systems with the intermediary metabolism of the gilthead sea bream (*Sparus aurata*). For this reason, the following compounds, related to both systems, were administered orally: i) T3, the biologically active thyroid hormone; ii) propylthiouracil (PTU), a thyroid hormone synthesis inhibitor; iii) cortisol, the main corticoid hormone in fish; and iv) dexamethasone, a synthetic glucocorticoid. After 35 days of treatment, various enzymatic activities related to the energy metabolism were analyzed in liver, white muscle and gills. Glycogen phosporilase (GP), lactate dehydrogenase (LDH), glutamate dehydrogenase (GDH) and 3-hydroxyacyl-CoA dehydrogenase (HOAD) activities pointed to a tight relationship between thyroid and interrenal systems, however several differences can be derived amongst them. The main result is that PTU stimulates lipid catabolism and amino acid turnover, as well as enhances glycogenolysis and anaerobic metabolism in some tissues, while T3 seems to increase only lipid catabolism at hepatic level. On the other hand, cortisol mainly enhances muscular catabolism while dexamethasone increases general catabolism. The differential responses in all the tissues to the compounds tested need further analysis in order to clarify the mechanisms involved in intermediary metabolism and its liaisons with the thyroid and stress axes.

Introduction

Fish thyroid physiology is complex and involves many different systems (metabolism, growth, osmoregulation, reproduction, amongst others), being different among vertebrates. Under physiological conditions, and regulated by the pituitary-secreted thyroid stimulating hormone, fish thyroid follicles secrete mainly, if not exclusively, thyroxine (T4) (1). This hormone is distributed by blood vessels to the target cells, where it can be deiodinated, producing the biologically active thyroid hormone, triiodothyronine (T3) (2). Thyroid hormones (THs) are involved in the control of many processes such as metabolism (3) or somatic growth (4), among others. Effects of THs on fish metabolism have frequently been studied, but a consistent picture could not be constructed as important differences between species appear to exist (5, 6).

The hypothalamus-pituitary-interrenal system produces corticosteroids (namely cortisol) in fish (7). Cortisol is considered to be the main stress hormone and it

promotes metabolic pathways that increase gluconeogenesis and glycogenolysis, as well as affects amino acid metabolism (8), thus stimulating catabolism of glycogen, lipids and protein (9).

Relationships between thyroid and interrenal axes have been described and seem to share controllers centrally (10). THs and cortisol trigger, alone or combined, the activation or inactivation of several energy metabolic enzymes (11). Anyway, metabolic responses to each hormone seem to vary widely among the different fish species, and no clear pattern of actions could be ascribed (3).

The aim of this study is, therefore, to try to clarify the effects of both axes on the energy metabolism in the teleost species gilthead sea bream (*Sparus aurata*). To achieve this, various compounds related to thyroid and interrenal systems were administered to the specimens, and their effects on several enzymatic activities related to carbohydrate, lipid and protein metabolism on different tissues were analyzed.

Material and Methods

Juveniles of gilthead sea bream (*S. aurata*) (n = 120, 18.1 ± 0.2 g weight and 9.9 ± 0.0 cm length) were distributed randomly in five 400 L tanks. Specimens were maintained in a flow-through system at 40 ppt of salinity, 19 ºC and natural photoperiod (fall in the south of Spain). Fish were fed with one of the following compounds: i) control; ii) 10 mg T3 kg food^{-1} (11); iii) 5 mg PTU kg food^{-1} (Ruiz-Jarabo, data not published); iv) 400 mg Cortisol kg food^{-1} (12); and v) 300 mg Dexamethasone (DXM) kg food^{-1} (13). Food preparation was conducted as described above (14). After 35 days, 12 animals per group were sampled. White muscle, liver and gills were collected. Glycogen phosporilase (GP) (15), lactate dehydrogenase (LDH) (16), glutamate dehydrogenase (GDH) (17) and 3-hydroxiacyl-CoA dehydrogenase (HOAD) (modified from 18) activities were analyzed.

Results and Discussion

Gilthead sea bream juveniles fed with T3, PTU, cortisol or DXM exhibit different energy metabolism effects. GP activity (Table 1) indicates glycogen consumption, and it is increased in the DXM group in every tissue sampled. This enzymatic activity is also stimulated when animals are fed with PTU and cortisol, but only in the muscle. LDH activity (Table 2) is enhanced in all experimental groups in the muscle, while cortisol and DXM stimulated the anaerobic metabolism also in gills. HOAD activity (Table 3) represents lipid catabolism. It is enhanced in the muscle by PTU, cortisol and DXM, in the liver by T3 and DXM, and in the gills by PTU and DXM. GDH activity (Table 4) increased in the muscle and gills when fish are treated with cortisol and DXM, which indicates protein catabolism. This enzymatic activity decreased in the liver of those groups, showing that the amino acids produced by this catabolic process are consumed in the same tissues where they are produced, indicating that the liver is not involved in protein catabolism. On the other hand, PTU increased GDH activity in muscle and gills, which is related to amino acid turnover and protein synthesis.

Table 1. Effect of T3, PTU, Cortisol and DXM after 35 days of treatment in *S. aurata* on the GP activity (U/mg prot) (mean ± SEM, n=8). * indicates differences with respect to control. # indicates differences T3-PTU or cortisol-DXM (Student´s-T test, p<0.05).

	Control	T3	PTU	Cortisol	DXM
Liver	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.4 ± 0.1 *#
Muscle	9.7 ± 0.9	12.1 ± 0.7	13.8 ± 0.5 *	13.8 ± 0.6 *	14.5 ± 0.7 *
Gills	1.9 ± 0.2	2.2 ± 0.3	2.0 ± 0.3	2.2 ± 0.2	3.3 ± 0.5 *#

Table 2. Effect of T3, PTU, Cortisol and DXM after 35 days of treatment in *S. aurata* on the LDH activity (U/mg prot). More details in Table 1.

	Control	T3	PTU	Cortisol	DXM
Liver	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0
Muscle	0.1 ± 0.0	0.2 ± 0.0 *	0.2 ± 0.0 *	0.3 ± 0.0 *	0.7 ± 0.1 *#
Gills	0.6 ± 0.0	0.5 ± 0.1	0.6 ± 0.1	1.3 ± 0.0 *	1.3 ± 0.1 *

Table 3. Effect of T3, PTU, Cortisol and DXM after 35 days of treatment in *S. aurata* on the GDH activity (U/mg prot). More details in Table 1.

	Control	T3	PTU	Cortisol	DXM
Liver	18.0 ± 1.2	19.3 ± 1.4	18.2 ± 1.5	12.6 ± 1.1 *	9.1 ± 0.9 *
Muscle	1.6 ± 0.1	1.8 ± 0.1	2.1 ± 0.1 *	2.2 ± 0.1 *	3.0 ± 0.2 *#
Gills	2.3 ± 0.1	2.6 ± 0.1	2.8 ± 0.1 *	2.4 ± 0.1	3.0 ± 0.1 *#

Table 4. Effect of T3, PTU, Cortisol and DXM after 35 days of treatment in *S. aurata* on the HOAD activity (U/mg prot). More details in Table 1.

	Control	T3	PTU	Cortisol	DXM
Liver	1.5 ± 0.1	1.8 ± 0.1 *	1.5 ± 0.1 #	1.5 ± 0.1	2.1 ± 0.1 *#
Muscle	0.6 ± 0.0	0.7 ± 0.0	0.7 ± 0.0 *	0.8 ± 0.1 *	1.3 ± 0.1 *#
Gills	1.3 ± 0.0	1.4 ± 0.1	1.5 ± 0.1 *	1.5 ± 0.1	1.9 ± 0.0 *#

In conclusion, PTU, which is supposed to act as a hypothyroid drug, stimulates lipid catabolism and amino acid turnover in muscle and gills, while it enhances glycogenolysis and anaerobic metabolism in muscle. T3 seems to stimulate only lipid catabolism at hepatic level. DXM increases general catabolism in all tissues tested, pointing to a hyper stimulation of glucocorticoid receptor(s), which leads to a chronic stress situation. Cortisol mainly increases muscular catabolism. More studies are necessary to fully understand the mechanisms of action of those axes in fish metabolism such as ammonia excretion pathways.

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BRAIN SEROTONERGIC AND DOPAMINERGIC SYSTEMS DURING THE STRESS RESPONSE IN RAINBOW TROUT: A TIME-COURSE STUDY

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The activity of the brain monoaminergic systems, including the noradrenergic, the serotonergic and the dopaminergic system, is usually increased shortly after exposure to stress, with the response of the serotonergic system being especially consistent among species and types of stressors. Once activated, the monoaminergic activity has several actions on activating other elements of the physiological cascade of the stress response. However, their involvement is complex and several other biochemical actors involved in the stress response, such as corticotropin-releasing factor (CRF) or cortisol, in turn have effects on the monoaminergic systems. Furthermore, states of chronic stress could induce important long-lasting changes in the monoaminergic systems. In an attempt to better characterize the response of the monoaminergic systems to stress in fish, we report here a time-course study of the activation of the serotonergic and dopaminergic systems after exposure to an acute stressor and its temporal relationship with the activation of other routine stress biomarkers such as plasma catecholamines, glucose, lactate and cortisol. The rapid serotonergic response after the exposure to stress suggests that the serotonergic tone could be important in the recognition of potential stressors by the central nervous system and act as a mediator between the perception of the stressor by the sensory organs and the primary release of catecholamines and corticosteroids to plasma.

Introduction

When exposed to a stressor, animals respond with a complex series of behavioral and biochemical mechanisms at different levels in order to be prepared to cope with any potential threat (1). The role of most of the elements participating in the stress response is well known, whereas the function of some others, such as the brain monoaminergic neurotransmitters is not yet clear. The activity of the central monoaminergic systems, including the serotonergic, dopaminergic and noradrenergic networks, usually increases after exposure to different types of stressors, such as handling, isolation, predator exposure, pollutant exposure or crowding (2-4), with the response of the serotonergic activity being especially consistent. However, the causes and consequences of those increased activities within the stress response along with the dynamics of the monoaminergic response to stress are not well known. Hence, the objective of the present study was to get a better knowledge about the timing of the response of the brain monoaminergic systems to stress. We investigated the activation of the brain monoaminergic systems after an acute stressor in rainbow trout together with the temporal relationship of that response in relation to the activation of other typically used stress markers such as the plasma catecholamines, glucose, lactate or cortisol.

Materials and Methods

In a first experiment, trout (80.6 ± 13.9 g body mass) were distributed (12 fish per tank) among 12 experimental tanks (80 liter tanks). After an acclimation period of 5 days, each of the following protocols was randomly assigned to two replicate tanks: No chasing (controls) and chasing for 15 seconds, 2 min, 5 min or 15 min. The fish were chased in their tanks with a small net for the stipulated time in each case. After the chasing protocol, fish were immediately anesthetized in-tank by adding 0.2% of 2-phenoxyethanol to the water. After circa 1 min of anesthetic exposure, five fish were removed from the tank for sampling. After blood extraction, the fish were sacrificed by spinal transection and hypothalamus and telencephalon were dissected out and stored in dry ice. Plasma was obtained after centrifugation of blood (6000 x g, 10 min, 4 °C). All fish were sampled within 3 min after the end of the chasing protocol.

In a second experiment, fish (90.7 ± 13.5 g body mass) were distributed (12 fish per tank) among 12 experimental tanks. After an acclimation period of 5 days, each of the following chasing protocols was assigned to two replicate tanks: No chasing (controls) or chasing for 5 min followed by a recovery period of 15 min, 45 min, 2 h, 4 h or 8 h. After the corresponding recovery period, fish were anesthetized, sacrificed and sampled as described for experiment 1.

Biochemical analyses: the levels of cortisol, glucose and lactate were evaluated in plasma with commercial kits. The plasma catecholamines, as well as the monoamine levels in the hypothalamus and the telencephalon, were analyzed by HPLC.

Results

As expected, cortisol, glucose and lactate levels increased after exposure to the stressor. Cortisol levels showed a steep increase with time after stress (picture not shown). A significant increase in cortisol levels was already observed after only 2 min of chasing and the maximum was observed after 15 min of chasing. At longer times, cortisol levels returned to control levels 8 hours after chasing. Similarly, glucose levels reached a maximum after 15 min of chasing and returned to control levels 8 hours after chasing. The lactate levels increased with chasing duration until the end of the first experiment and returned to control levels 4 hours after stress exposure.

The response of the plasma catecholamines occurred very quickly. Both adrenaline and noradrenaline were slightly increased after only 15 seconds of chasing and reached a maximum 2 minutes after the beginning of stress (Fig. 1). In the second experiment, both catecholamines showed signals of recovery at 45 min after chasing and had returned to unstressed levels 2 hours after the stress stimulus (Fig. 1).

Regarding the monoaminergic systems, the alterations induced by the chasing protocol affected mainly the serotonergic system and the alterations in the dopaminergic system were minor (data not shown). The serotonergic activity rapidly increased in both hypothalamus and telencephalon, as shown in the 5-hydroxyin-

dole-3-acetic acid (5HIAA)/serotonin (5HT) ratio (Fig. 2). The increase was obvious after only 15 seconds of stress. After that initial increase, the serotonergic activity remained above control levels without important changes until the end of experiment in both brain regions. At longer times, the serotonergic activity showed a maximum in both regions 45 min after stress. After that, the values started to decrease, returning to control values after 2 hours in the hypothalamus and 8 hours in the telencephalon (Fig. 2). In both regions, the increases in the serotonergic ratio were parallel to those in the levels of the metabolite 5HIAA whereas 5HT levels remained unaltered in all groups (data not shown).

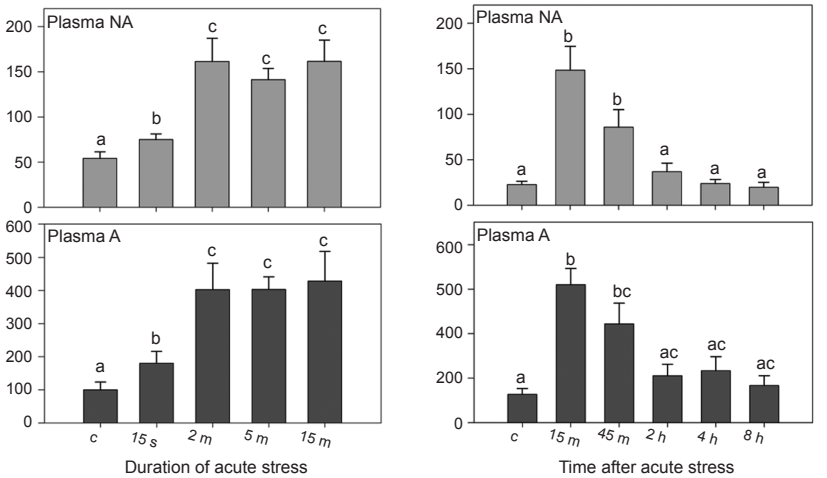

Figure 1. Plasma levels of the catecholamines noradrenaline (NA) and adrenaline (A) after exposure to an acute stress (chasing). A) Response during the first minutes after stress, B) Recovery during the first hours after chasing. Data are shown as mean ± SEM (n=10). Different letters indicate significant differences among groups (P≤0.05).

Discussion

While it has been shown that different kinds of stress stimulate the activity of central monoaminergic systems, especially the serotonergic system, the hierarchical position of these monoaminergic systems in the central regulation of the stress response is unclear. In our study, the acute stress protocol induced a very rapid change in hypothalamic and telencephalic 5HIAA/5HT ratio, an indicator of serotonergic activity, which increases in seconds, something that was observed before in lizards displaying elevated serotonergic activity only 30 seconds after a stressful social confrontation (5). The promoting effect of the acute stress on the serotonergic activity was transient, and serotonergic ratios went back to control levels in a few hours in a very similar way to plasma cortisol and plasma catecholamines. Interestingly, after only 15 seconds of chasing, the plasma concentration of catecholamines was already starting to increase, while the serotonergic ratio had already reached a plateau. Our results suggest that the response of 5HT activity occurs previously to or at least concomitantly with the rise of catecholamine levels in plasma. Therefore, besides its role in the regulation of the hypothalamus-pitu-

itary-interrenal axis, central 5HT activity could also play a role in triggering the brain-sympathetic nervous system-chromaffin cell axis, leading to the massive release of catecholamines to the blood. The initial activation of the brain monoaminergic neurons could be part of the stressor recognition mechanisms, taking part in the central integration of the stress signals to initiate the neuroendocrine response.

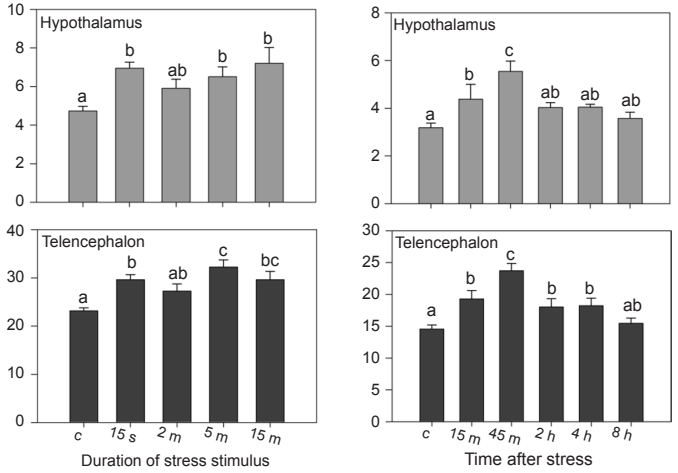

Figure 2. Serotonergic activity (estimated using the 5-hydroxyindole-3-acetic acid/serotonin ratio, 5HIAA/5HT) in the hypothalamus and telencephalon after exposure to an acute stress (chasing). A) Response during the first minutes after stress, B) Recovery during the first hours after chasing. Data are shown as mean ± SEM (n=10). Different letters indicate significant differences among groups (P≤0.05).

Acknowledgements

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CAN ANESTHETICS AVOID THE STRESS RESPONSE CAUSED BY PERSECUTION?: A COMPARATIVE ANALYSIS OF ESSENTIAL OIL OF *LIPPIA ALBA* AND 2-PHENOXYETHANOL ON STRESS RESPONSE IN THE GILTHEAD SEA BREAM (*SPARUS AURATA*)

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1. Department of Physiology and Pharmacology, Federal University of Santa Maria, Santa Maria, Rio Grande do Sul, Brazil. 2. Department of Biology, Faculty of Marine and Environmental Sciences, University of Cádiz, Puerto Real, Cádiz, Spain. 3. Institute of Marine Sciences of Andalucía (CSIC), Puerto Real, Cádiz, Spain. 4. Department of Industrial Pharmacy, Federal University of Santa Maria, Santa Maria, Rio Grande do Sul, Brazil.

The effects of the natural essential oil of *Lippia alba* (EOLA) and commercial anesthetic 2-phenoxyethanol (2-PHE) on stress response to persecution were investigated in the gilthead sea bream (*Sparus aurata*). Plasma cortisol levels, hypothalamic corticotrophin-releasing hormone (CRH) and corticotrophin-releasing hormone binding protein (CRHBP), as well as pituitary prolactin (PRL), growth hormone (GH), and both proopiomelanocortin (POMC) “a” and “b” expressions were determined in specimens submitted for four hours to one of the following treatments: control, EOLA (28 $mg \cdot L^{-1}$) or 2-PHE (39 $mg \cdot L^{-1}$), with or without stress induced by persecution. Stress induced by persecution enhanced plasma cortisol levels, decreased hypothalamic CRHBP and pituitary PRL expressions while stimulated pituitary GH expression. Exposure to EOLA increased plasma cortisol levels and diminished PRL expression. However, treatment with 2-PHE only stimulated pituitary POMC B expression without effect on the other parameters assessed. Finally, specimens submitted to stress by persecution with EOLA in the tank decreased CRHBP and increased POMC A expression, while no significant differences were observed with 2-PHE in any parameter assessed. Our results elucidated that persecution indeed is able to trigger stress response in the sea bream. Additionally, 2-PHE seems to be more effective than EOLA in preventing the effects of stress, at least at the concentration tested in our experiment.

Introduction

In aquaculture, fish are subjected to routine practices (i.e. handling, capture, biometrics, blood collection or transportation) that activate the stress system and may affect adversely their health and welfare (1). In an attempt to reduce the stress induced by these procedures, the use of anesthetics is advisable provided that adequate concentrations are established for a specific purpose, i.e. sedation or deep anesthesia (2, 3). Essential oil of *Lippia alba* (EOLA) has anesthetic and sedative properties for teleosts (4). Here we have analyzed the ability of two anesthetics [EOLA and 2-phenoxyethanol (2-PHE)] to prevent or mitigate the stress response in gilthead sea bream (*Sparus aurata*) specimens disturbed by persecution.

Materials and Methods

Gilthead sea bream specimens were distributed in the following groups: control, EOLA (28 mg·L^{-1}) or 2-PHE (39·mg L^{-1}), undisturbed or stressed by persecution every 50 min, for 4 hours. The anesthetic concentrations were determined in previous experiments (data not shown) and chosen because they did not cause deep anesthesia (5). Plasma cortisol levels were measured by indirect enzyme immunoassay (ELISA) as previously described (6). Expressions of hypothalamic CRH and CRHBP-mRNA, as well as pituitary POMCs, PRL and GH were analyzed by real-time-qPCR.

Results and Discussion

The rise of plasma cortisol levels in stressed fish revealed that the persecution used in the present study was effective as an stress model. Contrary to what was expected, the EOLA by itself increased the cortisol levels. However, sedation of fish with 2-PHE prevented cortisol rise (Fig. 1).

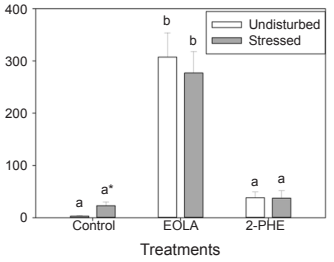

Figure 1. Plasma cortisol levels in *S. aurata* specimens exposed to EOLA or 2-PHE, undisturbed or stressed by persecution. Data are shown as mean ± SEM (n = 8). Different letters indicate difference between treatments (control, EOLA, 2-PHE) in the same group (undisturbed or stressed) ($P < 0.05$; one-way ANOVA, Tukey's test). Asterisks indicate difference from undisturbed group in the same treatment ($P < 0.05$; t-Student).

Hypothalamic CRH expression did not change in fish stressed or exposed to the anesthetics. Nevertheless, expression of CRH-BP was lower in stressed fish and fish exposed to EOLA with additional stress (Fig. 2). These results may be a consequence of the elevated cortisol levels, which act as a negative feedback regulator of hypothalamic-pituitary-interrenal (HPI) axis, at the level of the hypothalamus.

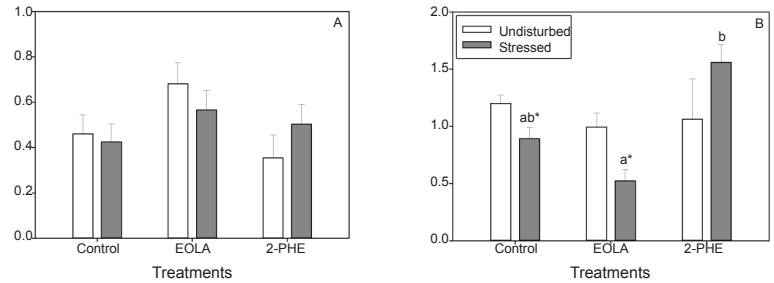

Figure 2. Hypothalamic CRH (A) and CRHBP (B) expression in *S. aurata* specimens exposed to EOLA or 2-PHE, undisturbed or stressed by persecution. Further details as in legend of Fig. 1.

Proopiomelanocortin (POMC) can be processed to adenocorticotropic hormone (ACTH) and melanocortin peptides (1). Stressed and exposed to EOLA, specimens displayed higher pituitary POMCa expression compared to fish submitted to EOLA only or stressed fish without substances added to water (Fig. 3A). This finding is in accordance with cortisol levels presented in Fig. 1. In the case of POMCb (Fig. 3B), the increased expression seen in fish exposed to 2-PHE both stressed and undisturbed could be mainly related with the melanocyte-stimulating hormone (MSH) instead of ACTH production, since these fish did not experience a rise in cortisol levels.

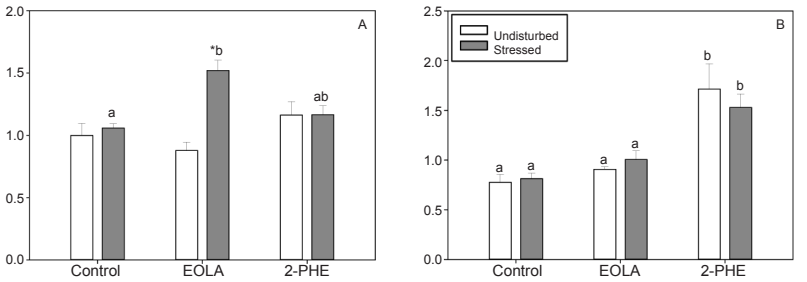

Figure 3. Pituitary POMCa (A) and POMCb (B) expression in *S. aurata* exposed to EOLA or 2-PHE, undisturbed or stressed by persecution. Further details as in legend of Fig. 1.

Persecution and sedation with both anesthetics reduced PRL expression compared to undisturbed control. On the other hand, applied stress (persecution) did not change PRL expression in specimens exposed to both EOLA and 2-PHE (Fig. 4A).

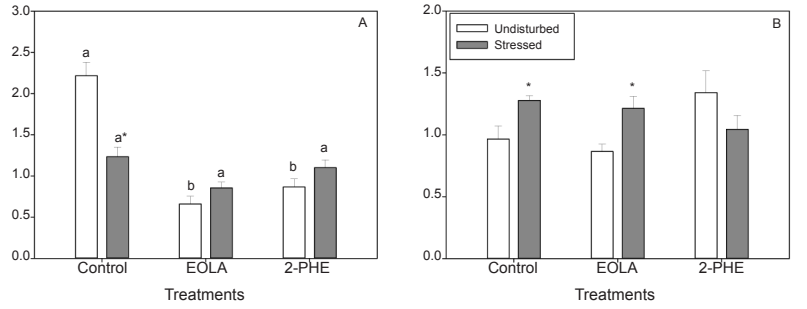

Figure 4. Pituitary PRL (A) and GH (B) expression in *S. aurata* specimens exposed to EOLA or 2-PHE, undisturbed or stressed by persecution. Data are shown as mean ± SEM (n = 8). Further details as in legend of Fig. 1.

Regarding GH, higher expression was detected in stressed fish of control and EOLA treatments compared with undisturbed fish of the same treatments (Fig. 4B). The increase in GH expression may be an indirect response resulting from the increased energy demand of any stress situation (1).

In conclusion, our results indicated that 2-PHE appeared to be more effective than EOLA in avoiding stress response caused by persecution in *S. aurata* specimens.

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THE STRESS RESPONSE AND OSMOREGULATION IN ANTARCTIC FISH *NOTOTHENIA ROSSII* AND *NOTOTHENIA CORIICEPS* EXPOSED TO THERMAL AND OSMOTIC CHALLENGES

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The ability of fish living in the waters around the Antarctic Peninsula to adapt to increases in temperature may be crucial for their survival as annual air and surface water average temperatures have increased by almost 3 °C in the last 50 years. These fish have developed structural, physiological and behavioral adaptations fitting a very cold marine environment, which questions such a possibility. The osmotic and stress parameters in two notothenid species from King George Island experimentally exposed to abrupt and gradual transfer to increased temperature and reduced salinity were evaluated. These fish appear to have unexpected thermal and osmoregulatory plasticity in the short-term, but our data suggests that in the long run their stress response may be compromised by the allostatic load posed by such challenges.

Introduction

Antarctic fishes evolved in a stable thermal and haline environment for 30 million years, and developed remarkable adaptions such as anti-freeze proteins, aglomerular kidneys and low metabolic rates. Recent climate changes contributed to a significant rise in water temperature and forecast models indicate the rate of such changes will increase in coming years, especially in the coastal areas of maritime Antarctica, possibly leading to ice melting and freshening of shallow waters in enclosed areas. Previous studies have suggested that several Antarctic fish species may have a subresponsive stress axis and that the very low water temperatures are responsible for reduced enzymatic activity and slower catecholamine release (1). In addition, it has also been indicated that heat-shock proteins (HSP), inducible in nearly every other species, are expressed constitutively in the cold-adapted fishes (2). Here we evaluated the cortisol response to increased temperature and reduced salinity in *Notothenia rossii* and *N. coriiceps*, two common species in the Northern Antarctic Ocean.

Materials and Methods

In experiments run in 2005, 2012 and 2013, we exposed two species of Nototheniidae to abrupt and/or rapid but gradual changes in water temperature or/and salinity. Fish collected around the Rothera (UK) and Arctowski (PL) stations, using traps or fishing poles respectively, were transferred to tanks in cold rooms. After a one week adjustment period at natural temperature and salinity (0-2 °C, 30-33‰), fish were 1) directly transferred to low and high temperatures or low salinities, and sampled at 3 and 24 hours; 2) gradually acclimated up to 10 days to -1, 4, 6 and 8 °C using thermostat-controlled heaters, and to 20 and 10‰ by adding fresh water to recirculating tanks. Blood was collected into heparinized syringes and the plasma sepa-

rated and frozen. Cortisol was assayed by RIA, osmolality determined using a vapor pressure osmometer and ions determined by flame photometry and a chloride analyzer. Gene expression was analyzed by qPCR in RNA extracted from liver samples.

Results and Discussion

1. Abrupt challenges. Rapid transfer of *N. rossii* to either a dilute or warmer medium did not produce any significant change in plasma osmolality (Fig. 1A) in relation to fish transferred to a control situation within 3 hours. However, after 24 hours, osmolality was significantly reduced in fish in the 10‰ but also in fish in full strength seawater warmed to 8° C. The handling and transfer procedures appear to have induced a rise in plasma cortisol (Fig. 1B), with values in almost all cases significantly higher at 3 hours than at 24 hours after transfer.

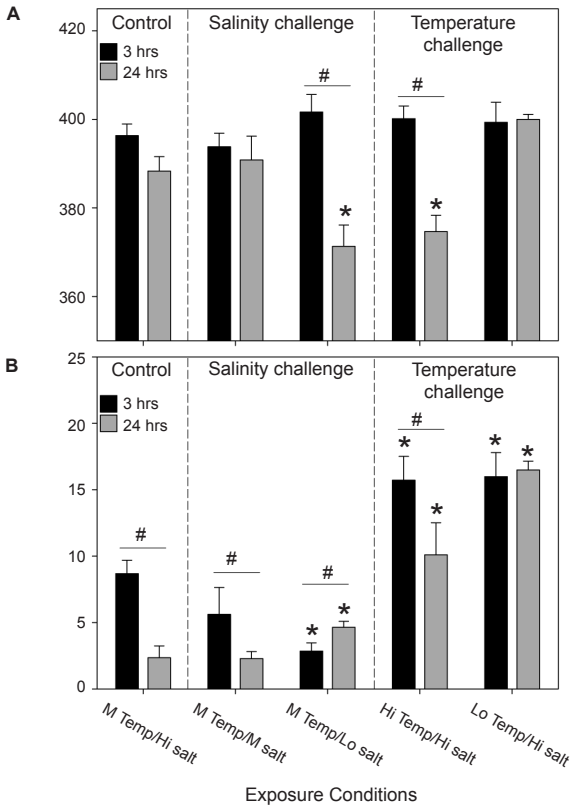

Figure 1. Plasma osmolality (A) and cortisol (B) in blood samples collected at 3 hours (black bars) and 24 hours (gray bars) after abrupt transfer from the control situation (M:1.5 °C and Hi:33‰) to diluted salinities (M:20‰ and Lo:10‰) at the control temperature or high (Hi:8 °C) and low (Lo: -1.0 °C) temperatures at the control salinity. Data are shown as mean ± SEM (n=6). *denotes differences from the respective time control, # indicates differences between time points (P≤0.05; one-way Anova).

It is noteworthy that while cortisol levels changed little or were slightly reduced after transfer to a reduced salinity , the short-term exposure to both high (8 °C) and low (-1 °C) temperatures had a significantly larger and longer influence on cortisol release. Nevertheless, the maximum cortisol levels measured are relatively low when compared to temperate species. No mortality was observed in either trial.

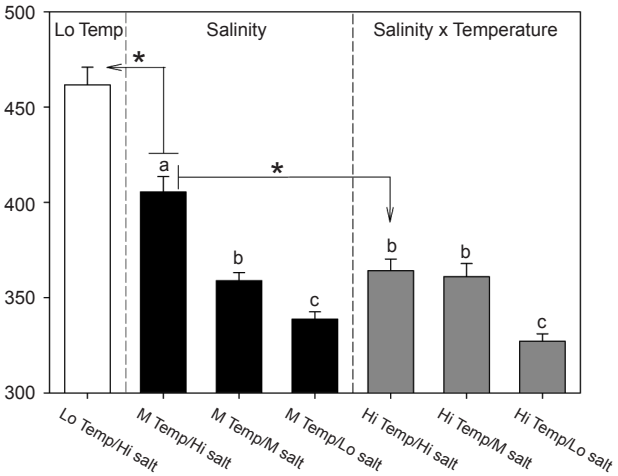

Figure 2. Plasma osmolality in *N. rossii* exposed for 5 days to gradual decreases in water salinity (from Hi:33‰ to M:20‰ or Lo:10‰) and increases in temperature (from M:1.5 °C to Lo:-1.0 °C and Hi:8 °C). Samples were obtained after 3 days in the final exposure condition. * highlights differences at same salinity and different temperatures. Different letters significant differences (n=6, P≤0.05; two-way ANOVA).

Table 1. Plasma sodium, chloride and potassium concentrations in *Notothenia rossii* in relation to gradual acclimation conditions.

Conditions	Temp	Salt	Na^+ (mM)	Cl^- (mM)	K^+ (mM)
Lo Temp/Hi Salt	**- 1.0 °C**	**30 ‰**	190.9 ± 6.17 ^{a}	178.3 ± 12.47 ^{a}	2.3 ± 0.33 ^{b}
M Temp/Hi Salt	**+ 1.5 °C**	**30 ‰**	187.2 ± 1.94 ^{a}	173.6 ± 2.39 ^{a}	3.0 ± 0.11 ^{a}
M Temp/M Salt	**+ 1.5 °C**	**20 ‰**	180.4 ± 1.16 ^{b}	165.8 ± 1.68 ^{b}	2.9 ± 0.12 ^{a}
M Temp/Lo Salt	**+ 1.5 °C**	**10 ‰**	173.3 ± 1.56 ^{c}	156.3 ± 2.10 ^{c}	2.5 ± 0.07 ^{b}
Hi Temp/Hi Salt	**+ 6.0 °C**	**30 ‰**	177.9 ± 1.86 ^{b}	161.9 ± 4.33 ^{b}	3.2 ± 0.17 ^{a}
Hi Temp/M Salt	**+ 6.0 °C**	**20 ‰**	176.7 ± 2.00 ^{b}	160.4 ± 2.50 ^{b}	2.9 ± 0.08 ^{a}
Hi Temp/Lo Salt	**+ 6.0 °C**	**10 ‰**	164.5 ± 2.80 ^{d}	148.9 ± 3.71 ^{c}	2.5 ± 0.09 ^{b}

Significant difference is denoted with different letters (P≤0.05; two-way ANOVA).

2. Gradual challenges. A gradual salinity reduction caused a reduction in osmolality that parallels changes in the medium (Fig. 2). However, the magnitude of the reduction was dependent on temperature (P=0.006). Thus while fish exposed to normal salinity and elevated temperature had significant lower osmolality than in control conditions, those exposed to colder water showed the highest osmolality.

Whereas the former may be explained by a temperature-induced increase in the activity of branchial iono-regulatory enzymes, as previously suggested (3), and corroborated by the sodium and chloride plasma levels (Table 1), the latter possibly reflects the combination of a reducing effect on metabolic rate and the presence of anti-freeze proteins, as total protein was high in the plasma of these animals (not shown).

The fish in this trial were not anesthetized for sampling, were chased in tanks with a net for up to 30 seconds and sampled 5-10 minutes later. This seemed to evoke a substantial rise in plasma cortisol in fish in normal conditions (Fig. 3A), not followed with the same intensity by any of the other experimental groups. It appears that the long-term exposure to demanding conditions may have reduced the immediate stress response and indeed fish in higher temperature conditions were less reactive to handling.

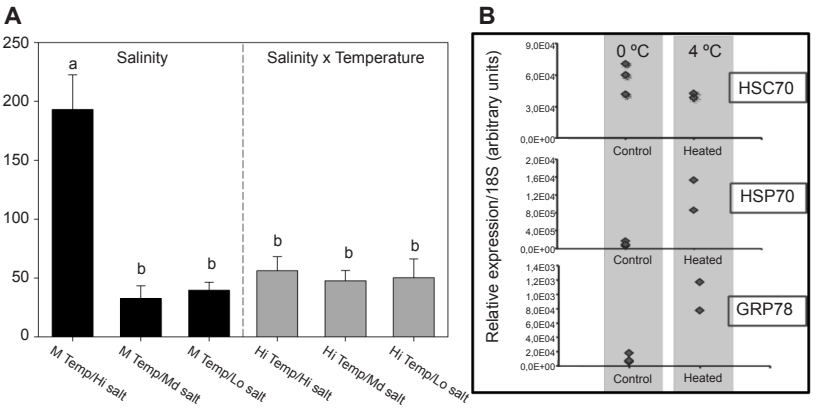

Figure 3. (A) Cortisol in plasma from *N. rossii* in conditions shown in fig. 2, collected after handling stress during sampling (n=6, P≤0.05; two-way ANOVA). (B) Expression of heat-shock proteins genes in the liver of *N. coriiceps* exposed to a gradual rise from 0 °C to 4 °C over 4 days (n=3).

3. Gene expression. In contrast to what was shown earlier for other notothenids (1), HSP70 and GRP(glucose responsive protein)78 appear to be up-regulated at higher temperatures in *N. coriiceps*, while HSC(heat shock cognate protein)70, constitutively expressed in mammals, shows no response (Fig. 3B). This may indicate that these fish, at the edge of the Antarctic Circumpolar Current, may not have such strict “Antarctic Physiology” as suggested for other species (1, 2, 3).

However, physiology may be jeopardized in warmer waters, as enhanced hypo-osmoregulation increases ionic gradients and thus energy expenditure. Both cortisol, and stress and metabolic proteins, were upregulated, but the immediate response was reduced in more demanding conditions. *N. rossii* and *N. coriiceps* are reactive to environmental changes/challenges, but their ability to accommodate rapid or adaptive responses may be compromised.

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ROLE OF GHRELIN SYSTEM COMPONENTS IN CUSHING'S DISEASE

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Cushing's disease is a result of hypercortisolism caused by a pituitary adenoma, in which corticotrope tumoral cells over-secrete adrenocorticotropin (ACTH). It has been described that ghrelin stimulates ACTH release from normal and tumoral corticotrope cells. Ghrelin is modified by the ghrelin O-acyltransferase (GOAT) enzyme, which is necessary to bind its only known receptor (GHS-R1a) and to exert the majority of its biological functions. There is also a truncated orphan variant of the receptor (GHS-R1b), which is expressed in a wide variety of tissues. We have recently discovered a novel human ghrelin variant (In1-ghrelin) that is over-expressed in some types of tumors. The aim of this study was to characterize the ghrelin system in human corticotropinomas (n=36) compared to normal pituitary (n=10), and to determine the functional role of In1-ghrelin in cultured cells derived from corticotropinomas. The use of quantitative real time PCR (qrtPCR) demonstrated a significant over-expression of In1-ghrelin and GHS-R1b in corticotropinomas, and a clear increase in GOAT mRNA levels. Additionally, In1-ghrelin expression, but not native-ghrelin expression, was positively correlated with GOAT, GHS-R1a and GHS-R1b expression. Furthermore, the expression of both receptors was positively correlated with the expression of POMC and CRH-R1. *In vitro*, native ghrelin and In1-ghrelin similarly stimulated free cytosolic calcium levels, a second messenger mediating hormone release. A significant over-secretion of ACTH was also observed after 24 h treatment with native-ghrelin and In1-ghrelin peptides. Of note, only In1-ghrelin evoked a slight, but significant, stimulation in proliferative rate in corticotropinoma cells. These results were also supported by the fact that In1-ghrelin transfection in cultured corticotropinoma cells increased cell proliferation com-

pared to mock-transfected cells. Altogether, our study provides novel findings regarding the role and potential clinical implications of the ghrelin system, especially In1-ghrelin, in the patho-physiology of human corticotropinomas.

Introduction

Cushing's disease is the result of excessive or dysregulated ACTH secretion by pituitary corticotrope tumoral cells (corticotropinomas; CTomas), which leads to a marked hypercortisolism that causes severe signs and symptoms such as emotional disturbance, moon facies, osteoporosis, hypertension, obesity, amenorrhea, etc. (1). Ghrelin is a hormone mainly produced in stomach which is well-known by its influence on several physiological processes including food intake, growth hormone release, gastric function, blood pressure, insulin secretion, etc. (2, 3). In addition, it has also been described that ghrelin stimulates ACTH release in healthy subjects (4, 5). In fact, previous studies from our group have demonstrated that ghrelin can act directly on CTomas derived from Cushing's disease patients to increase free cytosolic calcium concentration ($[Ca^{2+}]_i$) and that ghrelin is produced in CTomas cells and accumulated, together with ACTH, in secretory granules, suggesting a putative co-secretion of both factors (6).

The ghrelin family is a complex regulatory system comprised by several ghrelin-gene derived peptides, receptors and other factors. Specifically, the ghrelin gene gives rise to a pre-pro-peptide that produces several mature peptides including native ghrelin and obestatin (7). In addition, the ghrelin gene can also undergo processes of alternative splicing originating additional mRNAs such as the In1-ghrelin variant identified by our group (8), which is a variant that retains a short intron (In1) and encodes a different C-terminal tail than native ghrelin. Native ghrelin can be acylated in its serine 3 residue (Ser3) by the ghrelin O-acyltransferase (GOAT) enzyme, which is essential to bind to its cognate receptor, named GHS-R1a and, in turn, to exert the majority of its biological functions. The In1-ghrelin variant shares the Ser3 residue with native ghrelin, and, therefore, could be acylated by the GOAT enzyme. The endogenous receptor for In1-ghrelin variant is still to be identified. Interestingly, the ghrelin receptor gene encodes for an additional orphan, truncated receptor, GHS-R1b, expressed in a wide variety of tissues, whose presence may trap GHS-R1a in the endoplasmic reticulum, decreasing its presence in plasma membrane. In spite of the presence and potential importance of native ghrelin in CTomas derived from Cushing's disease patients, to date, no systematic, comprehensive studies have been performed to determine the effect of ghrelin system components (native ghrelin, In1-ghrelin, GOAT and receptors) in CTomas and, thus, to elucidate their functional role. Therefore, the main aims of this study were: 1) to characterize the ghrelin system in human CTomas compared to normal pituitaries (NPs); and 2) to determine the functional role of ghrelin system components in cultured cells derived from CTomas.

Materials and Methods

A series of human pituitary samples collected over the last 5 years was included in the study in which written informed consent was obtained from each patient. Specifically, the expression profile of 10 NPs and 36 CTomas was evaluated by

qrtPCR as previously described (9). Two NPs were obtained from a commercial source (pool of multiple individuals; CLONTECH; Palo Alto, CA), while the other eight were obtained from patients who went in for surgical removal of a pituitary tumor, but the piece of tissue obtained by our laboratory turned out to be NP tissue, as confirmed, as in the case of pituitary tumors, by the anatomo-pathologist. When available, remaining tissue from CTomas was used for functional assays. Specifically $[Ca^{2+}]_i$ kinetics, ACTH release and proliferation assays were performed *in vitro* using methods validated in our laboratory (6, 8, 9). Primary cells were treated with acylated (AG) and unacylated native ghrelin (UAG), and In1-ghrelin predicted acylated peptides (In1-97 and In1-98), synthesized in-house. Additionally, CTomas cell cultures were transfected with an In1-ghrelin plasmid, developed in our laboratory, to evaluate proliferative rate as previously described (8).

Results and Discussion

1. Expression profile. The use of qrtPCR analysis demonstrated the presence of all the components of the ghrelin system in CTomas and NPs as previously reported. Interestingly, a significant over-expression of In1-ghrelin and GHS-R1b was found in CTomas as compared with NPs, similarly to that previously observed in breast cancer samples (8). Moreover, a clear increase in native ghrelin, GHS-R1a and GOAT enzyme was also observed in CTomas as compared with NPs. The expression of In1-ghrelin was positively correlated to that of GOAT and GHS-R1b expression in CTomas, suggesting that In1-ghrelin might be also a substrate of GOAT enzyme. In addition, the expression of both GHS-R1a and GHS-R1b were positively correlated with the expression of POMC and CRH-R1 suggesting that the ghrelin system is functionally associated with key components of the regulation of the corticotrope function.

2. Direct effect of the ghrelin system on $[Ca^{2+}]_i$ kinetics and ACTH release. Intracellular calcium acts as a second messenger involved in hormone release in normal and tumoral pituitary cells. Previous results from our laboratory (6) revealed that AG increased $[Ca^{2+}]_i$, but in this report, evaluation of the effect of UAG and novel In1-ghrelin peptides was not carried out. The data of the present work indicate that UAG did not exert any effect in $[Ca^{2+}]_i$ but AG and In1-ghrelin peptides similarly elevated $[Ca^{2+}]_i$. Indeed, AG activated 74.1% of CToma cells, reaching 217.6% maximal response at 34.7s. In1-97 stimulated 43.9% of CToma cells, evoking a 187.5% maximal response at 44.9s while In1-98 activated 37.8% of the cells, evoking a 161.1% maximal response at 51.4s. These results suggest that the acylation of ghrelin peptides by GOAT is essential to exert calcium mobilization in CToma cells. In terms of ACTH release, AG as well as In1-ghrelin peptides (In1-97 and In1-98) clearly stimulated ACTH secretion *in vitro* in human corticotropinoma cells (209.2%, 160.7% and 177.1%, respectively) as compared to their respective controls after 24 h treatment. UAG did not exert any effect.

3. Effect of the ghrelin system on proliferative rate. AG and UAG did not significantly affect cell proliferation in CToma cell cultures. However, In1-ghrelin treatment evoked a slight, but significant, stimulation of proliferation in CToma cells. In addition, over-expression of In1-ghrelin in CToma cell cultures (transfected cells with In1-ghrelin plasmid) clearly increased the proliferation of CToma cells as compared with cells transfected with the mock-control plasmid.

Altogether, our results demonstrate that: 1) the ghrelin system is altered in human CTomas in that a general over-expression of all components, specially In1-ghrelin and GHS-R1b, was observed; 2) native acylated ghrelin as well as In1-ghrelin variant exert similar stimulatory effects on human CTomas in terms of $[Ca^{2+}]_i$ kinetics and ACTH release and; 3) only the In1-ghrelin variant is able to increase cell proliferation in CToma cells, suggesting that blockade of the In1-ghrelin variant might have some therapeutic benefit by decreasing both ACTH release and proliferation in human CTomas. These data provide novel avenues to investigate the precise patho-physiological role and potential clinical implications of this regulatory system, especially of the In1-ghrelin variant, in Cushing's disease.

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DIFFERENTIAL ROLE OF GHRELIN SYSTEM COMPONENTS IN HUMAN PROSTATE CANCER

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Prostate cancer is the most commonly diagnosed malignancy and the second leading cause of cancer-related deaths in the Western male population. In all cancers, aberrant and alternative splicing events generate proteins that could influence the tumor cell physiology and survival of patients. In1-ghrelin, a spliced ghrelin gene variant has been shown to be overexpressed in breast tumors, and to increase breast cancer cell lines proliferation. Recently, ghrelin gene expression has been found in normal and prostate carcinomas. We found that In1-ghrelin and GOAT (the enzyme responsible for ghrelin acylation) mRNA levels were significantly increased in human prostate tumors compared to normal prostate tissue while ghrelin mRNA levels were not altered. Comparably, In1-ghrelin and GOAT mRNA expression was found at detectable levels in all prostate tumor-derived cell lines studied (22Rv1, DU145, PC3, VCaP), while ghrelin expression was not present. Interestingly, In1-ghrelin treatment evoked a significant increase in the proliferation rate of DU145 and VCaP cells as compared with untreated-controls, while native-ghrelin peptide did not induce any significant response. The differential role of alternative spliced In1-ghrelin vs. native ghrelin in prostate cancer highlights the importance of this recently identified variant in tumor development and points out new clinical pathways in human prostate pathologies, both in early detection of the disease and possible therapeutic targets.

Introduction

RNA splicing is a tightly regulated process whereby nascent pre-mRNAs are modified by removing the introns and joining the exons (1). Most mammalian genes undergo alternative RNA splicing (AS), which increases the complexity of the genome by generating several mRNA variants from a single gene (1, 2). A fundamental characteristic of cancer cells is the modification of normal cellular processes, and deregulation of splicing is not an exception (3). Indeed, alternative and aberrant splicing events have been linked to malignant transformation, particularly those alterations occurring in genes associated with cancer susceptibility and/or progression (3, 4). Thus, in many types of cancers, these splicing alterations originate functionally significant biomarkers (5).

In particular, ghrelin is a peptide hormone that was originally isolated from rat stomach as the endogenous ligand for the growth hormone secretagogue receptor (GHSR1a) (6). Ghrelin needs to be acylated by the ghrelin-O-acyl transferase (GOAT) enzyme (7) in order to exert the majority of its functions, including the regulation of appetite and gut motility, modulation of growth hormone release from the anterior pituitary and roles in the cardiovascular and immune systems (8). Ghrelin and its receptor are expressed in a number of cancers and cancer derived cell lines and may play a role in processes associated with cancer progression, including cell proliferation, apoptosis, and cell invasion and migration (9). Yet the precise role of the ghrelin system in cancer is poorly understood. Notably, it is now been established that native ghrelin is not the only functional peptide derived from the ghrelin gene (10). Our and other laboratories have discovered the existence of several ghrelin-gene derived peptides which include the In1-ghrelin variant identified by our group in mice (11) and humans (12). Moreover, a truncated isoform of GHSR1a with 5TMD, the GHSR type-1b (GHSR1b) which does not bind ghrelin, is found in the many tumor types and cancer cell lines, however, its potential role in tumor regulation remains unknown. Recently, our group has shown the existence of a differential expression profile of the In1-ghrelin spliced variant compared to native ghrelin as well as of the GHSR1b in normal mammary gland tissues versus breast cancer tissues suggesting a potential patho-physiological role of these spliced variants in breast cancer (12).

Given the fact that prostate cancer, an endocrine, heterogeneous and complex cancer, has a high incidence in the male population, the aim of this study was to investigate the presence of the ghrelin system [native ghrelin, In1-ghrelin variant, ghrelin-O-acyl transferase (GOAT) and ghrelin receptors (GHSRs)] and the potential role of this system in prostate cancer using both human primary prostate samples and prostate cancer cell lines as models.

Materials and Methods

1. *Human biopsies*. Human prostate biopsies were collected at the Hospital Universitario Reina Sofia in Cordoba and placed at -80 ºC. Each sample was identified as normal or carcinoma by a pathologist. The study was approved by the Hospital and University Ethics Committee. Written informed consent was obtained from all patients. RNA was extracted using All Prep DNA/RNA/Protein Mini Kit (Qiagen). Total RNA (1 µg) was reverse transcribed (RT) using the cDNA First-Strand Synthesis kit with random primers according to the manufacturer's instructions (Fermentas, Hanover, MD, USA). A complete expression profile of the ghrelin system (native ghrelin, In1-ghrelin, GOAT and GHSR1a/GHSR1b) was determined by quantitative real-time RT-PCR (qrtPCR).

2. *Prostate cancer derived cell lines*. Both androgen dependent (22RV1, VCaP, LnCAP) and androgen independent (DU145, PC3) human prostate cancer derived cell lines were used. An expression profile of the ghrelin system (native ghrelin, In1-ghrelin, GOAT and GHSR1a/GHSR1b) was determined in all prostate cancer cell lines as indicated above.

3. *Proliferation*. Each prostate cancer cell line was treated with the ghrelin-gene derived peptides (native ghrelin or In1-ghrelin derived peptides named In1-97 and

In1-98). Proliferation was measured at different times (24, 48 and 72 h) using the MTT method which is a colorimetric assay where MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is reduced to purple formazan in living cells. Absorbance of this colored solution was measured by the FlexStation System 3 (Molecular Devices).

Results and Discussion

1. In1-ghrelin is overexpressed in human prostate cancer. In1-ghrelin and GOAT (the enzyme responsible for ghrelin acylation) mRNA levels were significantly increased in human prostate tumors (n=33) compared to normal prostate tissue (n=7), while native ghrelin mRNA levels were not altered. Ghrelin receptors (GHSR1a/1b) expression was not detected at significant levels. These results are consistent with previous results from our group showing that, in other pathologies such as breast cancer (12) and pituitary tumors (unpublished data: see chapter of Ibáñez-Costa et al., in this book), In1-ghrelin and GOAT are significantly overexpressed as compared with normal tissues. Therefore, the data presented herein suggest that the overexpression of some components of the ghrelin system could be a common feature in a number of endocrine-related cancers, where they might be exerting an important role as regulators of cancer development and progression.

2. In1-ghrelin and GOAT are expressed in a wide variety of prostate cancer cell lines. In1-ghrelin and GOAT expression was detected in all the androgen-dependent or androgen-independent prostate cancer cell lines studied. However, expression of native ghrelin as well as ghrelin-receptors was almost negligible. This data indicate that prostate cancer derived cell lines closely resemble the expression pattern observed in *in vivo* prostate tumors and, therefore, could be a suitable model to analyze the role of ghrelin system components in this pathology.

3. In1-ghrelin derived peptides increase proliferation in prostate cancer cell lines. Treatment with In1-ghrelin derived peptides (In1-97 and In1-98), but not with native ghrelin, clearly increased the proliferation of both, androgen dependent (VCaP) and independent (DU145) prostate cell lines, suggesting that In1-ghrelin could play a pathophysiological role in the development and progression of prostate tumors.

Altogether, our results provide primary evidence that In1-ghrelin is a potential novel element of the ghrelin family with a likely pathophysiological role in human prostate function, since it is over-expressed in prostate cancer, wherein it can regulate tumoral cell proliferation.

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REGULATION OF ALTERNATIVE SPLICING OF THE GHRELIN GENE BY THE ANTISENSE STRAND GENE GHRLOS

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The ghrelin is a pleiotropic hormone encoded by a gene (GHRL) that can also originate several splicing variants associated with diverse neuroendocrine pathologies. Specifically, our group has recently identified a new splicing variant named In1-ghrelin, which is differentially expressed in several tumoral pathologies, where it could be directly related to their tumorigenicity. However, the molecular mechanisms responsible for the appearance of this variant and its regulation are unknown. In this regard, many studies point out the important role of non-coding RNAs in the regulation of alternative splicing processes. Particularly, the existence of a non-coding gene (GHRLOS) in the antisense strand of GHRL has recently been described. Specifically, GHRLOS presents six known splicing variants, which could be involved in regulating the alternative splicing of GHRL. Some of those variants overlap with the intron retained in the In1-ghrelin mRNA variant and could, therefore, be associated with its generation. Hence, we aimed to determine: 1) the relationship between the expression of each GHRLOS variant, ghrelin and In1-ghrelin in human tissues and in endocrine-related tumors; 2) the regulatory capacity of GHRLOS variants on ghrelin and In1-ghrelin expression in tumoral cell lines; and 3) the effect of GHRLOS variants overexpression on functional parameters, such as proliferation, in tumoral cell lines. Our data demonstrate that the expression of some GHRLOS variants is differentially correlated with the expression of the ghrelin gene variants (i.e. the correlation between the expression levels of GHRLOS-2 and GHRLOS-3 with In1-ghrelin is R^2=0,964 and R^2=0,807, respectively, in human tissues). Additionally, overexpression of GHRLOS-1 variant induced an increase in the proliferation rate of prostate tumoral cell lines similar to that shown for In1-ghrelin overexpression. Altogether, our data suggest an involvement of GHRLOS variants in the alternative splicing of the GHRL and, consequently, in the dys regulation of the ghrelin system observed in many endocrine-related cancers.

Introduction

The ghrelin gene has been widely studied during the last decade. It was originally reported that the human pre-pro-ghrelin polypeptide precursor was encoded by a single-copy gene (GHRL) that is located in the short arm of chromosome 3 and was organized into four coding exons (exons 1-4) (1). However, it is now known

that the human ghrelin gene contains a number of upstream first exons (exon -1, exon 0 and extended exon 1) in addition to the four pre-pro-ghrelin coding exons (2), which can act as alternative sites for transcription initiation; this therefore, drastically increases the complexity of the gene. Indeed, soon after the discovery of ghrelin, a number of alternative ghrelin gene derived peptides and mRNA splice variants were identified by a number of independent laboratories (3). One of these splicing variants is the In1-ghrelin variant, which arises as a result of the retention of the intron-1 (4). Interestingly, this variant shares the N-terminal region with native ghrelin, but due to the change in the reading frame originated by the intron retention, it has a completely different C-terminal region. In1-ghrelin variant has been shown to be associated with the patho-physiology of some types of cancers, such as breast cancer, where it may affect the tumor malignancy. In addition, its over-expression in breast cancer cell lines markedly increases proliferation (4).

Among the systems frequently associated with the splicing process, it is worth mentioning the non-coding RNAs (ncRNAs), which have been shown to be involved in the regulation of several systems and signal responses (5) and, to be associated with a growing number of diseases (6). In the case of the ghrelin gene, the existence of an antisense gene named ghrelin opposite strand or GHRLOS, which is located in the opposite strand of the ghrelin gene and encodes a number of naturally occurring ncRNAs has recently been discovered (7). Therefore, GHRLOS variants might be involved in the regulation of the alternative splicing of GHRL. Moreover, since some of the GHRLOS variants overlap with the intron retained in the In1-ghrelin mRNA variant, the GHRLOS variants could also be specifically involved in the generation and regulation of the In1-ghrelin variant.

Hence, in the present study we aimed to determine 1) the relationship between the expression of each GHRLOS variant, native-ghrelin and In1-ghrelin variant in human tissues and in endocrine-related tumors, 2) the regulatory capacity of GHRLOS variants on ghrelin and In1-ghrelin expression in tumoral cell lines and, 3) the effect of GHRLOS variants overexpression on functional parameters, such as proliferation, in tumoral cell lines.

Materials and Methods

1. Samples and cell lines. The correlations between mRNA expression levels of the GHRLOS variants with other components of the ghrelin system were conducted in a wide variety of commercially available human tissues included in the ClonTech human tissue RNA battery “Human Total RNA Master Panel II”. In addition, two human prostate cancer cell lines (PC3 and Vcap; ATCC, Manassas, VA) were maintained in RPMI 1640 Medium (Lonza) or in Dulbecco’s Modified Eagle Medium (DMEM), respectively, with 1g/L glucose (Lonza) and supplemented with 10% fetal bovine serum (FBS), 1% antibiotic-antimycotic (100X solution, 10,000u/ml penicillin, 10mg/ml streptomycin and 25mg/ml amphotericin B, Sigma), and 2mM L-glutamine at 37 ºC and 5% CO_2.

2. RNA isolation and reverse transcription. Nucleic acids were isolated with Tryzol (Invitrogen) following the manufacturer’s instructions and treated with DNAse for removing genomic DNA. Total RNA (1mg) was reverse transcribed (RT)

with the cDNA "First-Strand Synthesis Kit" using random primers according to the manufacturer's instructions (Fermentas) and as previously described (4).

3. Primer design. Primers used for quantitative real-time RT-PCR (qrtPCR) of GHRLOS variants as well as for generating the cloning vectors of the GHRLOS variants were designed using the human GHRLOS variants sequences (NR_004431.2, NR_024144, NR_024145.1) as template and the primer3-software (http://frodo.wi.mit.edu) following the guidelines previously described by our group (4).

4. qrtPCR. All qrtPCR assays were run with 25ng of cDNA using a 2x IQ-SYBR-green mastermix (BioRad) and were carried out in an iCycler IQ thermal cycler (BioRad) as previously reported (4).

5. Plasmid and transfection. GHRLOS variants were cloned from the ClonTech human tissue RNA battery in an expression vector [T-Vector pMBL (MolBioLab Cat No MBL097)] and transfected in VCap and PC3 human prostate cancer cell lines using Lipofectamine-2000 (Gibco) following the manufacturer's recommendations.

6. Proliferation assay. The proliferation studies were carried out using the MTT assay (CN-M5655, Sigma) following manufacturer's recommendations. Assay measurements were performed on a Flex-Station system.

Results and Discussion

1. RNA expression levels and correlations. qrtPCR studies were performed in order to identify possible associations between the mRNA levels of GHRLOS variants with other ghrelin gene-derived variants. This analysis revealed a clear positive correlation between GHRLOS variants 1, 2 and 3 and In1-ghrelin mRNA expression levels (R^2=0,944, R2=0,964 and R^2=0,807, respectively and a p-value < 0,001 in all the cases). However, this correlation was not observed with native ghrelin. Therefore, these results could suggest a possible specific regulatory role of some of these GHRLOS variants in the alternative splicing of the GHRL gene in order to induce the generation of the In1-ghrelin variant.

2. Proliferation assay and changes in mRNA expression levels. Taking into account the association between the expression levels of GHRLOS variants and In1-ghrelin, we cloned the GHRLOS variants GHRLOS-1 and GHRLOS-3 in the expression vector, T-Vector pMBL, with the aim of studying the effects its overexpression on: 1) proliferation of human prostate cancer cell lines, by transfecting the cloned GHRLOS variants into these cells; and/or 2) the expression levels of In1-ghrelin and native ghrelin.

- In the case of GHRLOS-3 over-expression, we did not observe changes in either In1-ghrelin or native-ghrelin mRNA expression levels. Accordingly, transfection with GHRLOS-3 did not induce any change in the proliferation of these prostate cancer cell lines.

- In addition, a proliferation study in cells transfected with GHRLOS-1 variant showed that this variant induced a clear increase in proliferation be-

tween 30 and 50% at 24, 48 and 72 hours. The increased proliferation rates could be associated with an increased expression of In1-ghrelin, in that over-expression of the In1-ghrelin variant has been shown to increase proliferation rate in this cell line (4).

These data indicate the existence of a clear correlation between the expression levels of three GHRLOS variants and In1-ghrelin, but not with native ghrelin, which would suggest a potential regulatory role for GHRLOS variants on In1-ghrelin expression. In addition, the fact that overexpression with GHRLOS-1 variant, but not with GHRLOS-3 variant, induced an increase in the proliferation of human prostate tumor cell lines suggests a variant-specific function and, probably, regulation in human prostate cancer cell lines. Altogether, these results suggest a possible patho-physiological relevance of the GHRLOS gene in the cross-talk between the ghrelin system components and prostate cancer development and/or progression, which add a new level of complexity in the regulation of the ghrelin system.

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ROLE OF NEW COMPONENTS OF SOMATOSTATIN AND GHRELIN SYSTEMS IN THE PROLIFERATION OF PANCREATIC NEUROENDOCRINE TUMOR CELL LINES

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Somatostatin (SST), cortistatin (CORT), their receptors (sst1-5), ghrelin and its receptors (GHSRs) comprise two interrelated systems that regulate multiple cell functions from hormone secretions to proliferation. Some components of these systems are co-expressed in several tissues, such as the pancreas. Specifically, SST, CORT, ghrelin and some of their receptors (sst1, sst2, sst5 and GHSRs) are expressed at pancreatic level. Recently, our group has identified new functional components of these systems in humans which are originated by alternative splicing as is the case of two truncated variants of sst5 (sst5TMD5, sst5TMD4), as well as an alternative ghrelin gene variant, named In1-ghrelin. These new components of SST/CORT and ghrelin systems have been found to be altered in several endocrine-related pathologies including pituitary tumors and breast cancers, where their overexpression was associated with increased malignancy or tumorigenesis. The fact that our results indicated that the expression of these human splice variants is also altered in neuroendocrine tumors (NETs) compared to normal tissue, led us to hypothesize that these new variants could also be implicated in malignancy of pancreatic NETs. In the present study, we analyzed the mRNA expression profile of both, SST/CORT and ghrelin, axes in cell lines derived from pancreatic NETs (BON-1 and QGP-1) and determined the functional consequences of the over-exposure to these new variants (human In1-ghrelin and truncated sst5) on cell proliferation. Our results showed that the majority of components of SST/CORT and ghrelin systems are present at detectable levels in BON-1 and QGP-1, although at variable expression levels. Interestingly, overexpression of In1-ghrelin, sst5TMD5 or sst5TMD4 markedly enhanced the proliferation rate in both cell lines, suggesting that these new functional variants could play a relevant role in the patho-physiology of human pancreatic NETs.

Introduction

SST/CORT and ghrelin systems comprise two families of peptides that regulate multiple cellular functions such as hormone secretions and proliferation. SST and CORT act through a family of five receptors with seven transmembrane domains

named sst1-5 (1, 2). Ghrelin is a peptide that can be acylated by the ghrelin-O-acyl transferase (GOAT) enzyme, a modification that is essential to bind to its receptor, GHSR1a (3). SST, CORT and ghrelin, as well as some of their receptors, are expressed in many endocrine tissues including the pancreas. Recently, new functional components of these systems originated by alternative splicing have been identified by our group; specifically, in the SST/CORT system, two truncated variants of the human sst5 (sst5TMD5 and sst5TMD4) (4) and, in the ghrelin system, an alternative ghrelin isoform, named In1-ghrelin, which retains an intron within its sequence (5). These new components have been found to be overexpressed in some types of tumors, being associated with an increase in malignancy or tumorigenesis (4-7; unpublished data: see chapter by Ibáñez-Costa et al., and Hormaechea-Agulla et al., in this book). In line with this, neuroendocrine tumors (NETs) are rare, aggressive and slow-growing tumors in which biological therapy such as SST analogs is usually, but not always, effective in controlling hormone production and proliferation (6). In a series of NETs collected by our group, it has been observed that both sst5 truncated variants are present in human NETs, sst5TMD4 always being more expressed than sst5TMD5, suggesting a putative role of these receptors in these pathologies (unpublished data). Moreover, In1-ghrelin was observed to be more expressed in NETs samples than in normal tissue, exhibiting a higher expression in progressive tumors than in stable tumors as well as in metastatic tumors than in non-metastatic counterparts. These results led us to hypothesize that these new splice components of the SST/CORT and ghrelin systems might play unique patho-physiological functions in human NETs which could have clinical repercussions and therefore, may possibly be used as potential therapeutic targets in the future.

Materials and Methods

To test the hypothesis, two cell lines derived from pancreatic human NETs (BON-1, QGP-1) were studied by analyzing the expression profile of SST/CORT and ghrelin systems as well as the functional consequences of the over-exposure to these splicing variants (the truncated sst5 variants and In1-ghrelin variant) on cell proliferation by overexpressing (transient transfection) the sst5TMD5 and sst5TMD4 or by treating the cells with In1-ghrelin peptides (In1-97 and In1-98).

Results and Discussion

1. SST/CORT and ghrelin systems are expressed in pancreatic neuroendocrine tumor cell lines. Several components of both systems were present in the two human cell lines, the expression of GOAT enzyme and SST being markedly high. Interestingly, native ghrelin was expressed in BON-1 cells, but not in QGP-1. When comparing SST receptors, sst5 was the dominant receptor in both cell lines while the expression of truncated sst5 receptors was not detected; making these cells a suitable experimental model to perform overexpression studies with truncated sst5 variants.

2. The over-expression of truncated sst5 variants increases the proliferation rate of NET cell lines. As expected, the transfection experiments with the truncated sst5 variants resulted in significantly higher mRNA levels of sst5TMD5 and sst5TMD4 in both cell lines. Functionally, the overexpression of sst5 variants sig-

nificantly increased the proliferation rate at different times, in both cell lines. This increase in the proliferation rate suggests that the presence of these variants induces an aggressive and proliferative behavior in these NET cells.

3. Over-exposure to the In1-ghrelin variant stimulates the proliferation rate in NET cell lines. NET cell lines were treated with In1-ghrelin derived peptides as well as with the native acylated and non-acylated ghrelin peptides. The results of this assay indicated that all these peptides stimulate proliferation rate at 48 h and 72 h.

4. Concluding remarks. The data presented in this study suggest that human truncated sst5 receptors (sst5TMD4 and sst5TMD5) are overexpressed in metastatic NET tissues and their overexpression exerts a potent stimulatory effect on cell proliferation in NET cell lines. Moreover, our data indicate that the In1-ghrelin variant is over-expressed in aggressive NET tumors that cannot be controlled with therapy, and that In1-ghrelin treatment increases proliferation in human NET cell lines. The results of this study could have a potential translational component, such as changes in the regulatory system comprised of SST and ghrelin, particularly the newly identified truncated sst5 and In1-ghrelin variants, could contribute significantly to the cellular and molecular deregulation associated with neuroendocrine tumors, and this information, especially from a patient-personalized perspective, may offer new tools to identify and develop original molecular targets for the diagnosis and/or therapeutic treatment of these pathologies in the future.

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GUT MOTILITY IN GOLDFISH (*CARASSIUS AURATUS*): ROLE OF DOPAMINE

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Dopamine (DA) is recognized as a neurotransmitter of the enteric nervous system, regulating digestive processes and intestinal motility in mammals. However, there is no such evidence to date in fish gut. A previous report shows that DA is acetylated in the intestine of goldfish (*Carassius auratus*) *in vitro*, suggesting possible functions of DA at this location. Therefore, the aim of the present study was to investigate the possible role of DA on gut motility in goldfish, and deep into the receptors and the intracellular signaling pathways involved. We used an *in vitro* organ bath system, coupled to an isometric force transducer for studying DA effect on gut motility. Conventional PCRs were carried out to identify the distribution pattern of DA receptor subtypes in different layers of the intestinal bulb. A biphasic effect of DA was observed *in vitro* inducing gut contraction at low concentration (1μM), and relaxations at higher concentrations (10 and 100 μM). To identify the possible receptors involved in the DA relaxation effect, we used a specific antagonist for D_1 (R(+)SCH23390) and D_2 (domperidone) dopaminergic receptors, and antagonists for α- and β- adrenergic receptors (yohimbine and propranolol, respectively). The relaxation induced by DA was totally blocked by the D_1 receptor antagonist. Moreover, we found that D_{1a1}, D_{1b}, D_{1c1}, D_{2a}, and D_3 receptor subtypes are differently distributed in the muscular-serosal and mucosa-submucosal layers. The relaxing effect of DA was not blocked by the inhibitors of the following enzymes: nitric oxide synthase (Nω- Nitro-L-arginine methyl ester, L-NAME), adenylyl cyclase (2', 3', dideoxyadenosine, DDA) and guanylyl cyclase (1H-[1,2,4] oxadiazolo[4,3-a]quinoxalin-1-one, ODQ). Finally, this DA effect seems to be on muscle cell directly, since it is insensitive to a voltage-dependent Na^+ channel blocker (tetrodotoxine). These results show the first evidence of DA as a neurotransmitter in the enteric nervous system that regulates gut motility in fish via specific receptors.

Introduction

In mammals and birds, DA is involved in the regulation of gastrointestinal functions mediated by the enteric nervous system, such as gut motility (1, 2), with stimulatory or inhibitory effects depending on the concentration, intestinal tract region and the species. The expression of dopaminergic receptors in different intestinal layers has been studied only in mammals (1). The possible relevance of DA in intestinal motility regulation in fish has recently been suggested in the goldfish gut (3). DA, at 1μM concentration, induces gut contraction in the *in vitro* system (3). The present study aimed to characterize the role of DA in gut motility in goldfish, and to explore the receptors and mechanisms underlying such action.

Materials and Methods

To characterize the putative effect of each drug on intestinal motility, fish were sacrificed 1 h after feeding, and the whole gut was rapidly removed and the foregut was prepared and mounted in an organ bath system as previously described (3). Intestinal strips were mounted longitudinally and attached to an isometric force transducer (LCM Systems Ltd., Cibertec, Madrid, Spain). For each gut preparation, a first trial with acetylcholine (10 μM) was performed to determine the maximal contraction, as a control of a proper response (3).

To determine the distribution pattern of dopaminergic receptors expression in the intestinal bulb, this gut region was extracted and separated into two layers: the muscle with the adherent myenteric plexus (MMP), and the mucosa-submucosa containing the submucosal plexus (MCP). Total RNA from goldfish tissues was extracted with Trizol (TRI® Reagent method, Sigma-Aldrich, Madrid, Spain) and treated with DNase (Promega, Madison, USA). Then, 1 μg of RNA from the intestine were retro-transcribed using the SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, USA). The tissular distribution of the following DA receptors subtypes (D_{1A1}, D_{1B}, D_{1C}, D_{2A}, D_{2B1}, D_{2B2}, D_3, D_4, D_{2B1S} and D_{2B1L}) was studied by PCR carried out in a 25 μl final volume containing: 1.25 U of *Taq* DNA Polymerase recombinant, PCR Buffer (20 mM Tris-HCl, 50 mM KCl pH 8,4), 1.5 mM of $MgCl_2$ (all from Invitrogen, Carlsbad, USA), 1 mM of primers (Sigma-Aldrich, Madrid, Spain) for D_1-like, D_{2B2}, D_3 and D_4 receptors or 0.4 μM of primers for the rest of D_2-like receptors; and 2 and 6 μl of cDNA for intestine and pituitary gland, respectively.

Results and Discussion

1. Biphasic response to DA in gut motility of goldfish. A non-cumulative concentration-response curve in isolated intestine shows a biphasic effect of DA, causing small contractions at lower concentrations (0.3 and 1 μM), and relaxation at higher concentrations (10 and 100 μM) respect to the basal tone (Fig. 1). This result agrees with that previously described for mammals and birds (2, 4). The relaxing or contracting effect of DA reported in mammals seems to depend mostly on the region of the digestive tract studied. Contractions often occur in proximal parts of the gut (2, 4), while the relaxation effect is observed in proximal and distal regions (4, 5).

2. D_1-like receptors mediate DA effects on gut motility in goldfish. The DA-induced relaxation of gut motility was blocked by preincubation with a specific D_1-like receptor antagonist (R(+)SCH23390), but not by a specific D_2-like receptor antagonist (domperidone) (Fig. 2). These results agree with those previously described in mouse ileum (5) and colon (6) showing that DA-induced relaxation was mediated through D_1-like receptors also in mammals. To discard the possible cross-binding of DA with adrenergic receptors, we preincubated foregut strips in the presence of α- or β- adrenergic receptors antagonists (yohimbine and propranolol, respectively) for 5 min before addition of DA to the medium. The relaxing effect of DA was not attenuated or blocked by any of these drugs. The effectiveness of these adrenergic antagonists in goldfish gut was demonstrated by the blocking of noradrenaline-induced relaxation (data not shown).

In addition to the motility results above described, we have confirmed the presence of D_1-like and D_2-like receptors in different layers of goldfish gut. We identified the subtypes D_{1A1}, and D_{1C} in the MCP layer of the goldfish gut, while the D_{1A1} and D_{1B} subtypes were found in the MMP layer. Regarding D_2-like receptors, the subtype D_{2A} was found only in the MCP layer, while D_{2B1}, D_{2B2} and D_4 were absent in both the MCP and MMP layers. Expression of the D_3 subtype was observed in both studied layers. These results support the specificity of the DA effect on gut motility, being mediated by the specific binding to its own receptors. Only one previous report in mice has described the location of DA receptors in intestinal layers (1). In this rodent, D_1, D_3 and D_5 receptors are expressed in both nerve-containing layers and the mucosa. In addition, D_2 receptor transcripts in mice seem to be restricted to the neurons, whereas the transcript for D_4 is restricted to the mucosal layer (1).

3. DA-induced relaxation is not mediated by adenylyl cyclase activation or by nitregic signaling pathway. Nitric oxide is an important relaxing agent in the smooth muscle of vertebrates, including fish (7). A nitrergic inhibitory tone was observed in the gut of different teleosts (7), including goldfish (8). Nitric oxide, in both nerve and muscle cells, can activate the downstream of cGMP, and both cAMP and cGMP can target some steps of the contractile signaling pathways, being able to induce relaxation. Therefore, in order to investigate if these signaling pathways could be involved in the DA relaxing effects on foregut motility in goldfish, the intestinal strips were pre-incubated for 5 minutes before DA addition to the bath with the inhibitor of adenylyl cyclase (DDA, 10μM), the nitric oxide synthase inhibitor (L-NAME, 100μM), and the guanylyl cyclase inhibitor (ODQ, 1μM). None of these inhibitors were able to block DA-induced relaxation (Table 1), suggesting that this smooth muscle relaxation is not caused by nitric oxide liberation, nor mediated by cAMP or cGMP synthesis. In line with our results, L-NAME did not cause any change in DA-induced relaxation in mouse ileum (5), however, conversely to our results, DDA was able to reduce the relaxation caused by DA, suggesting a participation of the cAMP pathway in DA-induced relaxation in this rodent.

To go more deeply into the signaling mechanism underlying the relaxing effect of DA on gut motility, we explored the possible activation of other interneurons, or alternatively the possible location of DA receptors directly on the smooth muscle. We used the voltage dependent Na^+ channel blocker, tetrodotoxin, to determine if enteric transmission is required in the DA relaxing effect. The pre-incubation of foregut with TTX did not block DA relaxing effect (Table 1) supporting the idea that DA could induce relaxation through D_1-like receptors located directly in the smooth muscle of foregut in goldfish.

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DISTINCT EVOLUTION OF PUTATIVE GHRELIN AND RELATED RECEPTORS IN NEMATODE AND ARTHROPOD GENOMES

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Human rhodopsin G-protein coupled superfamily receptors (GPCRs) have an important role in feed intake and energy expenditure. Sequence and function homologs of these receptors have been identified in the nematode *Caenorhabditis elegans* and in the arthropod *Drosophila melanogaster* suggesting that the endocrine mechanisms that regulate food intake emerged early and are conserved. Nematodes and arthropods are successful animal phyla characterized by a large number of representatives and their genomes have been modified by pressure generated by adaption to their environment during evolution. Previous studies from our lab demonstrated that nematodes and arthropods have a similar GPCR gene repertoire, but that receptor gene evolution was distinct and species-specific. It was hypothesized that this may be associated with their diverse life styles and feeding habits. In this study, putative ghrelin and related receptors in nematodes and arthropods were compared to human receptors and their emergence and evolution characterized. Understanding the evolution of feed intake regulation and the role of ghrelin and related receptors may provide new insights into control of feeding disorders and diseases that use insects as vectors.

Introduction

Food intake is essential for survival. In vertebrates, the mechanisms that regulate appetite and feeding behavior are tightly regulated by the endocrine system and involve members of the rhodopsin G-protein coupled receptor (GPCRs) family. GPCRs are the largest and most diverse group of membrane receptors and are characterized by the presence of 7 transmembrane domains and are highly conserved across metazoans. Recently, a comprehensive descriptive *in silico* analysis of nematodes and arthropods rhodopsin-GPCR that are homologs of human receptors involved in feeding was published by our group (1). In nematodes and arthropods, rhodopsin GPCRs appear to have undergone different evolutionary trajectories and gene expansions were observed in the genomes of free-living nematodes in comparison to parasitic species; and in arthropods, species-specific receptor gene deletions and duplications occurred. The difference in receptor gene number and distinct receptor family evolution was hypothesized to be a consequence of their diversity in life-styles and feeding habits. The present work describes the evolution of invertebrate homologs of human Ghrelin receptor GPCR subfamily and related members. The ghrelin receptor is also known as growth hormone secretagogue receptor (GHSR) and is related to GPR39 (Obestatin receptor) and Neuromedin U receptors (NMURs). In vertebrates, ghrelin stimulates growth and potently induces feed intake and weight gain and obstatin and NMURS are involved in reduction of feeding (2, 3). In invertebrates, homologs of the human GHSR/GPR29/NMUR system are poorly characterized with the exception of

the model organisms *C. elegans* (nematode) and *D. melanogaster* (arthropod). The function of the GHSR/GPR29/NMUR system differs between model nematodes and arthropods: in *C. elegans* they are involved in regulation of feeding (4) and in *D. melanogaster* they function in diuresis and rhythmic motor activity (5, 6). The aim of the present study was to reanalyze the evolution of the metazoan GHSR and related receptors using the data retrieved from nematodes and arthropods and compare them with humans.

Materials and Methods

A total of 7 nematodes and 5 arthropods genomes were explored; detailed description of the method for data retrieval are given in (1). Predicted conserved seven transmembrane regions (7TM) of GHSR/GPR29/NMUR GPCRs from human, nematodes and arthropods were retrieved from (Ensembl (http://www.ensembl.org/index.html) and Ensembl Metazoa (http://metazoa.ensembl.org/index.html). TM domains were concatenated and aligned using ClustalW and the manually edited alignment used to build the phylogenetic tree. The best model for tree construction was selected in ProtTest (2.4) according to the Akaike Information Criterion (AIC) and the WAG+G+F model was chosen. Phylogenetic trees were constructed using both the Neighbor Joining (NJ) and the Maximum Likelihood (ML) methods with 1000 and 100 bootstrap replicates, respectively. Both methods generated similar tree topologies. NJ analysis was performed in Mega5 and ML in the Phylogeny.fr platform (http://www.phylogeny.fr/). The consensus ML is presented and was constructed using a fixed proportion of invariant sites, 4 gamma distributed rate categories (gamma shape parameter=1.41) and built using the PhyML program (v3.0 aLRT). The resulting tree was edited in Inkscape program (version 0.48).

Results and Discussion

1. The nematode and arthropod putative GHSR/GPR39/NMUR members. In nematode and arthropod genomes, homologs of the human GHSR/GPR39/NMUR receptors were identified. In *C. elegans*, four NMUR-like receptors and two novel receptors (npr-20 and npr-21) were identified.. Searches in non-model nematode genomes revealed a similar gene repertoire to *C. elegans* (Table 1). The exception was *B. malayi*, where no receptors were found and this may be a consequence of its incomplete genome annotation. Comparisons across nematodes revealed that a higher receptor gene number (6) is present in free-living nematode genomes compared to parasitic nematodes and in *P. pacificus* and *M. incognita* only 3 and 4 receptors were identified, respectively, and in the worm *T. spiralis* only a single receptor gene was retrieved (Table 1).

In *D. melanogaster* the CapaR and three pyrokinin receptors exist and *in silico* searches identified a new putative receptor member (CG34381), which is similar to the *C. elegans* npr-20 and npr-21. A similar gene repertoire was found in the genome of the mosquitoes, *A. gambiae* and *A. aegypti*. However in the bee *A. mellifera*, 5 receptors were retrieved and in *B. mori* and *I. scapularis* only 2 receptors were identified.

Table 1. Putative GHSR/GPR39/NMUR receptor gene members in nematode and arthropod genomes.

Nematodes	Genes	Arthropods	Genes
Caenorhabditis elegans (Cel)	6	*Drosophila melanogaster* (Dme)	5
Caenorhabditis briggsae (Cbr)	6	*Anopheles gambiae* (Aga)	4
Caenorhabditis japonica (Cja)	6	*Aedes aegypti* (Aae)	4
Haemonchus contortus (Hco)	5	*Apis mellifera* (Ame)	5
Pristionchus pacificus (Ppa)	3	*Ixodes scapularis* (Isc)	2
Meloidogyne incognita (Min)	4	*Bombyx mori* (Bmo)	2
Brugia malayi (Bma)	ni		
Trichinella spiralis (Tsp)	1		

2. Evolutionary analysis. Phylogenetic analysis with putative GHSR/GPR39/NMURs receptors in the nematode and arthropod and the human homologs revealed that they shared common ancestry and that two major clusters exist (Fig. 1). One cluster contains the human receptors with the nematode and arthropod sequence homologs and a second cluster is nematode and arthropod exclusive. Within the first cluster, the human NMUR and GPR39 receptors tend to group with the four nematode NMUR-like receptors and no arthropod representatives were identified. In contrast, the human GHSR tends to cluster with the arthropod PK and capa receptors and no nematode receptor representative was found. The invertebrate exclusive cluster contains the nematode homologs of the *C. elegans* npr-20 and npr-21 and the *Drosophila* CG34281, for which homologs were identified in other arthropod genomes, but were not included in this study. In addition, within arthropods, species-specific receptor gene duplication events occur while in nematodes single homologs of the four *C. elegans* receptors were identified in other nematode genomes. Evolutionary analysis confirms that distinct evolutionary selective pressures affected the members of this family in the nematode and arthropod radiation and that specific gene duplications and deletions occurred in the two phyla. The existence of an invertebrate exclusive receptor group suggests that a specific gene duplication of the bilateria genome ancestral GHSR/GPR39/NMURs molecule occurred in the nematode and arthropod lineages after the protostome-deuterostome divergence. Further studies are needed to fully understand the distinct evolutionary profile of their members in the metazoa genome and to investigate how this has affected receptor function and their role in the regulation of feeding.

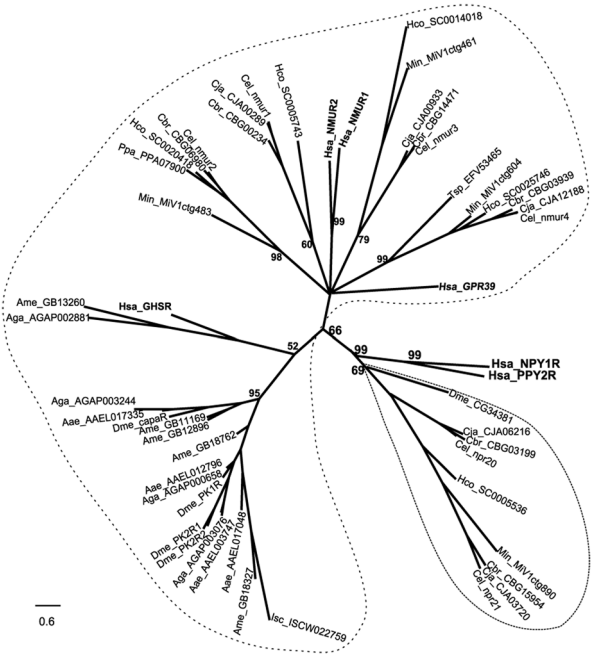

Figure 1. Phylogenetic relationship of metazoa GHSR/GPR39/NMUR receptor family members. The phylogenetic tree was constructed with the ML method and 100 bootstrap replicates and tree branches with bootstrap values <50% were collapsed. The human neuropeptide Y receptors (NPYRs) were used as an outgroup.

Acknowledgements

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EFFECTS OF TEMPERATURE ON GLOBAL DNA METHYLATION DURING EARLY DEVELOPMENT IN EUROPEAN SEA BASS

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Persistent epigenetic marks arise from early developmental environments and correlate with relevant biological processes later in life. Fish represent an effective animal model for the purposes of studying these epigenetic marks, such as DNA methylation. Recently, we showed that adult European sea bass (*Dicentrarchus labrax*) exposed to high temperature during early development show higher levels of gonadal aromatase promoter DNA methylation than fish raised in natural temperatures. However, the effect of different temperatures and the sensitivity of distinct early developmental stages of the larvae remain unclear. In the present study, global DNA methylation patterns were measured by Methylation-Sensitive AFLP (MSAP), which allows the comparison of genome-wide DNA methylation profiles between treatments. Two types of comparisons by MSAP were studied in European sea bass sampled 15 days post fertilization: (1) three groups of larvae that were exposed to low (13.5 ^{o}C), medium (17.5 ^{o}C) or high (19.5 ^{o}C) temperature, and (2) four groups of larvae that were either exposed to constant temperature (low or high) or that experienced a switch in temperature 5 days post fertilization, from low to high or vice versa. In the first comparison, significant differences in global DNA methylation patterns were detected among the temperature groups. In the second comparison, there was significant differentiation in DNA methylation between the four groups, as well as between groups compared pairwise. The differentiation was greater between larvae which experienced constant temperature during their lifetime and larvae which experienced a switch of temperature at 5 days post fertilization. Together, these results reveal that temperature changes from 0–15 days post fertilization strongly affect the methylome of the European sea bass.

Introduction

Epigenetic changes are mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence (1). Through epigenetic mechanisms, the information received from the genotype and the environment is integrated and produces a given phenotype, an effect which could be either short-term or long term (2). One of the main epigenetic mechanisms is DNA methylation, which is related to the control of gene expression. We have recently showed that temperature affects the DNA methylation in adult European sea bass (*Dicentrarchus labrax*) and more specifically, that fish treated with high temperature early during development show higher levels of DNA methylation in the promoter of the gonadal aromatase than fish treated with natural temperatures (3). In this study, we have focused on the effects of temperature on global DNA methylation early during development and especially at the beginning of the thermosensitive period.

Materials and Methods

1. Experiment 1: 0-15 dpf. Firstly, a temperature-response experiment was performed using fish raised at either natural (13.5 ºC), intermediate (17.5 ºC) or high (19.5 ºC) temperature and sampled at 15 dpf. Secondly, a time-response experiment was performed using 4 groups of fish: (1) raised at natural temperature (13.5 ºC), (2) raised at high temperature (19.5 ºC), (3) submitted to a switch from natural to high temperature at 5 dpf and (4) submitted to a switch from high to natural temperature at 5 dpf.

2. Experiment 2: 25 to 60 dpf. Fish raised at natural temperature were divided in 2 groups at 25 dpf and treated with either natural (17 ºC) or high temperature (21 ºC) until sampled at 60 dpf.

All groups of fish were run through the Methylation Sensitive Amplification Polymorphism (MSAP) protocol (4) resulting in electropherograms, which were verified, scored and statistically analyzed (5). For experiment 2, the expression of 8 genes related to epigenetic functions was tested by qPCR.

Results and Discussion

1. Experiment 1: 0-15 dpf. All 3 groups of fish submitted to different temperatures (temperature-response) showed significant differences in global DNA methylation profiles as detected by analysis of molecular variance (AMOVA; Table 1). In the time-response experiment, AMOVA detected significant differences between all 4 groups tested, as well as between groups when compared pairwise (Table 2), while the differentiation was stronger between larvae which experience constant temperature during their lifetime, and larvae that experienced a switch of temperature at 5 dpf.

Table 1. AMOVA results for the temperature-response experiment. Phi_ST values and the associated *P*-values are given for all three groups of high (HT), intermediate (IT) and natural (NT) temperature, as well as when compared pairwise.

	Phi_ST	***P*-value**
Total	0.1778	<0.0001
HT - IT	0.2003	<0.0001
IT - NT	0.08828	=0.0051
NT - HT	0.2309	<0.0001

Table 2. AMOVA results for the time-response experiment. Phi_ST values and the associated *P*-values are given for the pairwise comparison of the 4 groups: natural temperature (NT-NT), natural to high temperature (NT-HT), high to natural temperature (HT-NT) and high temperature (HT-HT), as well as for the comparison of all 4 groups.

	Phi_ST	***P*-value**
Total	0.194	<0.0001
HT-HT vs HT-LT	0.2367	<0.0001
HT-HT vs LT-HT	0.231	<0.0001
HT-HT vs LT-LT	0.1018	=9e-04
HT-LT vs LT-HT	0.1089	=0.0024
HT-LT vs LT-LT	0.2165	<0.0001
LT-HT vs LT-LT	0.2499	<0.0001

2. Experiment 2: 25-60 dpf. The 2 groups of fish submitted to different temperature from 25 to 60 dpf showed no significant difference in global DNA methylation profiles as detected by AMOVA (Table 3). From the 8 genes tested by qPCR, the DNA-methyltransferase 1 is found significantly less expressed in fish raised at high temperature (Figure 1).

Table 3. AMOVA results for the experiment 2, comparing natural and high temperature groups.

Phi_ST	P-value
0.01673	=0.1698

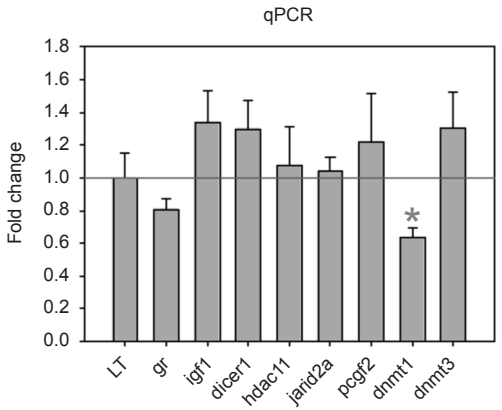

Figure 1. Gene expression results measured by qPCR on 8 genes related to epigenetic functions shown as fold change of the high temperature (HT) compared to the natural (LT) temperature group. The red line indicates the threshold marked by the LT group and the asterisk that there is significantly lower expression of the *dnmt1* in the HT group..

Together, these results reveal that temperature changes from 0-15 days post fertilization strongly affect the methylome of the European sea bass. The lack of significant differences in gene expression for most of the analyzed genes may be due to the fact that this was measured right after the temperature treatment but when the fish were still very small. It also remains to be determined whether such changes in DNA methylation are maintained and, more importantly, what genes remain permanently affected in their expression levels as a consequence of these epigenetic modifications.

Acknowledgements

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FOXL2 AND FOXL3 IN TELEOSTS: ORIGIN, REGULATION AND ROLE IN REPRODUCTION

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In mammals, the forkhead transcription factor Foxl2 is involved in the regulation of gene expression at different levels of the brain-pituitary-gonad (BPG) axis. However, little information is available regarding teleosts where two paralog genes, *foxl2a* and *foxl2b*, have been described. In this report, we suggest that these two genes should be renamed *foxl2* and *foxl3*, respectively, as they originated from an ancient non-teleost-specific whole genome duplication. In the European sea bass, *foxl2* is expressed along the BPG axis with higher levels in the ovary than in the testis. Gonadal expression through an annual reproductive cycle together with primary culture transfection studies suggest that *foxl2* could be involved in maintenance of ovarian identity, folliculogenesis, steroidogenesis and gonadotropin receptor regulation, while *foxl3* could be related to male specific genes and *Ihr* regulation. We tested the effect of estradiol on the expression of *foxl2* in vitro, but contrary to what was suggested on other reports on teleosts, we were not able to demonstrate any interaction.

Introduction

Foxl2 is a forkhead transcription factor that has been widely studied in mammals as it is associated with a genetic disease in humans that can lead to reproductive failure. It is involved in several biological processes such as development, ovarian maintenance, folliculogenesis and steroidogenesis (1). Initially, two *foxl2* teleost-specific paralogs were cloned in rainbow trout (*Onchorynchus mykiss*), namely *foxl2a* and *foxl2b* (2). It was suggested that these two genes originated during teleost-specific whole genome duplication (WGD). Since then, many studies in teleosts have focused on Foxl2a/*foxl2a* expression and function (3), while data about *foxl2b* are very scarce (2). We cloned these two genes in European sea bass (*Dicentrarchus labrax*) and this report summarizes our study of their origin. We also suggest new putative functions based on tissue expression and transfection of a primary culture of ovarian follicular cells. Finally, we have investigated the regulation of these two genes using in vitro models.

Materials and Methods

1. Synteny analyses were carried out using information from Ensembl (http://www.ensembl.org) on genome assemblies of human (*Homo sapiens*) GRCh37, chicken (*Gallus gallus*) WASHUC2, coelacanth (*Latimeria chalumnae*) LatCha1 and other available species. AnnotationDraftV1 assembly from the sea bass genome database (Kuhl & Reinhardt, unpublished) was used for this species.

2. RNA was extracted from different tissues of adult male and female sea bass for cDNA synthesis.

3. cDNA samples from ovary and testis reaching their first year of sexual maturation were used from a previous work to study gonad expression through a reproductive cycle (4).

4. Ovarian follicular cells were extracted and transiently transfected for 72 h with pcDNA3 (control), pcDNA3-*foxl2a* or pcDNA3-*foxl2b* (modified from 5). The same protocol was used to extract ovarian follicular cells that were immediately stored in liquid nitrogen. Once thawed, cells were cultivated for 24 h. Unsettled cells were removed and the medium changed. After 24 h, cells were stimulated with estradiol (50nM) or with diluted ethanol as control. RNA was sampled for qPCR expression analysis.

Results and Discussion

1. The syntenic analysis (Fig. 1) presents a highly conserved pattern for both *Foxl2*/*foxl2a* (A) and *foxl2b* (B). *foxl2*/*foxl2a* is present in all the species studied (data not presented): *foxl2b* could not be found in several species, such as human or *Xenopus tropicalis*, but is present in several non-teleost species, such as chicken or coelacanth. These findings are in agreement with a recent work published during the preparation of this report (6) and with our phylogenetic analysis (data not shown). Therefore, we suggest the renaming of *Foxl2*/*foxl2a* and *foxl2b* as *foxl2* and *foxl3* respectively, as the letters a and b used in the former names related to an origin in teleost-specific WGD.

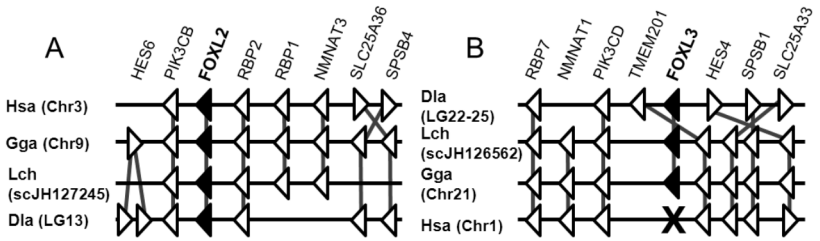

Figure 1. Syntenic alignment of chromosomic regions around *foxl2* (**A**) and *foxl3* (**B**) genes. Both are presented as black triangles. Chromosome segments and genes are represented as thick lines and triangles, respectively, indicating orientation of the transcription unit. Orthologous genes are linked with a grey line. A black cross represents the absence of *foxl3* in human species. Indicated genes are not necessarily contiguous. Dla, *D. labrax*; Gga, *G. gallus*; Hsa, *H. sapiens* and Lch, *L. chalumnae*.

2. Tissue expression. *foxl2* is expressed in all BPG axes in both male and female sea bass (Fig. 2), while *foxl3* expression was relatively more restricted. This expression pattern suggests that foxl2 could be involved in reproduction at several levels of the BPG, as described in mammals. The sex dimorphic pattern is very strong, as we observed higher *foxl2* expression levels in the ovary than in the testis. On the other hand, *foxl3* expression is higher in the testis than in the ovary.

3. Gonad expression through a reproductive cycle. *foxl2* expression in the ovary (Fig. 3) is higher during late vitellogenesis and ovulation, as is the case for

gondadotropin receptors (*fshr* and *lhr*), steroid acute regulatory protein (*star*) and aromatase (*cyp19a1*) gene expression (4). Expression levels of these four genes were increased after overexpression of sea bass *foxl2* in a primary culture of ovarian follicular cells (data not shown). Taken together these data suggest that in the ovary *foxl2* could be involved in steroidogenesis and regulation of gonadotropin receptors. *foxl3* presents an opposite pattern of expression with lower levels during mature stages and ovulation. This gene also presents strong variations in the testis with higher levels during early gametogenesis, which decrease through testis maturation. These data, together with other literature reports (2) and overexpression of *foxl3* in a primary culture of ovarian follicular cells (data not shown), suggest that *foxl3* could also be involved in regulation of male specific genes and *lhr* regulation. *foxl2* expression in the testis did not show any variations through the reproductive cycle (data not shown).

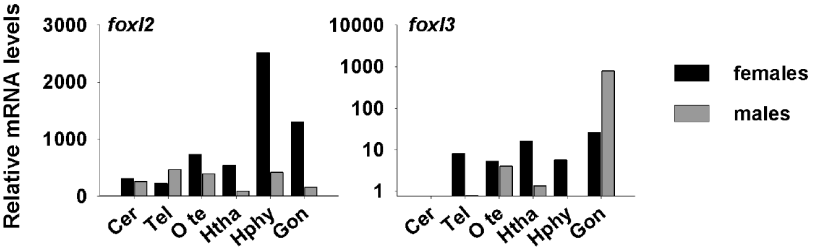

Figure 2. Expression of *foxl2* and *foxl3* in adult sea bass along the BPG axis. Each bar represents a pool of two animals. Tissue samples: Cerebellum, Telencephalon, Optic tectum, Hypothalamus, Hypophysis, Gonads.

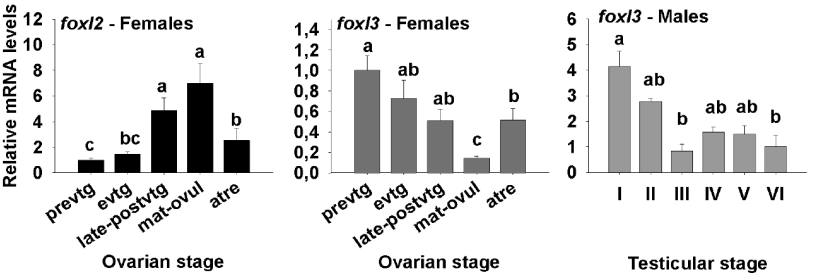

Figure 3. Expression of *foxl2* and *foxl3* in adult gonads through an annual reproductive cycle. Female stages: previtellogenesis, early vitellogenesis, late/postvitellogenesis, atresia. Male stages: immature (I), early-, mid-, late-recrudescence (II, III, IV), full spermiating (V), post-spawning (VI). Statistical differences between groups are indicated with letters above the bars ($p < 0.05$).

4. Regulation of *foxl2* by estradiol. Stimulation of ovarian follicular cells with 50nM estradiol (Fig. 4), a low pharmacological dose, increased *lhr* expression as described in the literature (7). However, in these conditions *foxl2* expression was not modified. The same experiment was repeated with 10nM estradiol (a high physiological concentration) with the same results (data not shown). In order to verify if oocyte-follicular layer communication was critical for *foxl2* up-regulation by estradiol, we stimulated ovarian tissue *in vitro* with 1nM and 1µM estradiol.

After 24 h stimulation we could not detect any variation in *foxl2* expression, while *lhr* expression was enhanced with 1μM estradiol (data not shown). In various teleost species it has been shown that estradiol causes up-regulation of *foxl2* expression (2) but, to our knowledge, this process has not been demonstrated to be direct. Our results demonstrate that in sea bass ovarian tissue, estradiol does not up-regulate *foxl2* expression.

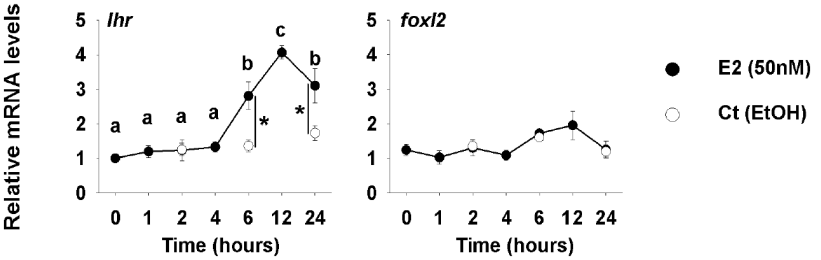

Figure 4. Effect of estradiol on primary culture of ovarian follicular cells. Statistical differences within the estradiol (E2) treated group are indicated with letters above the bars. Significant differences between E2 and control (Ct) groups are indicated with an asterisk ($p < 0.05$).

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THE KISSPEPTIN SYSTEM IN THE CONTROL OF THE REPRODUCTION IN VERTEBRATES: THE CASE OF THE EUROPEAN SEA BASS (*DICENTRARCHUS LABRAX*)

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In the last few years, studies about kisspeptins in mammals have revealed the important role of the *kiss*/*gpr54* pair as a key regulator of reproduction, including normal fertility and puberty. Kisspeptins are considered the most potent stimulator of gonadotropin secretion that has been discovered in all species. Recent studies have evidenced the existence of a variable number of *kiss* genes and kisspeptin receptors in non-mammalian vertebrates. Their physiological relevance, functions and signaling pathways still remain to be clarified according to species, gender and reproductive status. However, these genes have a conserved expression in brain and gonads across vertebrate species, thus suggesting a putative functional role in control of reproduction. In the case of the European sea bass, two *kiss* genes (*kiss1*, *kiss2*) and two kisspeptin receptors (*gpr54-1b*, *gpr54-2b*) have been identified. In the present work, we have studied the expression profiles of all four kisspeptin system genes and other reproductive related genes (gnrh-gonadotropin system) in the brain of adult male and female sea bass during their first reproductive cycle.

Introduction

The European sea bass is an important and highly-prized marine species in aquaculture, which has two *kiss* genes (*kiss1*, *kiss2*) and two kisspeptin receptors (*gpr54-1b*, *gpr54-2b*) (1, 2). Newly, the localization of the main brain nucleus where these genes are expressed has been characterized by using *in situ* hybridization and inmunohistochemistry techniques, thus correlating the expression of the ligands with their receptors (3, 4). In the present work, we studied the mRNA expression profiles of two *kiss*/*gpr54* systems, as well as *gnrh-I*/*gnrhr-II-1a* and gonadotropin genes (*fshβ* and *lhβ*) in the brain of adult male and female sea bass during different gonadal stages by quantitative real-time PCR (qRT-PCR).

Materials and Methods

Adult male (2 years old) and female (3 years old) sea bass were periodically sampled through their first reproductive cycle. Fish were sacrificed and gonads were collected to elucidate the testicular and ovarian stage of animals in each sampling point (n=6-16 males and 4-8 females per sampling) (5). The gonadosomatic index (GSI) was calculated according to the formula: gonad weight/body weight X 100. We dissected the brain, and separately collected the hypothalamus and pituitary and stored at -80 ºC. Total RNA was extracted using Maxwell 16LEV simplyRNA tissue kit (PROMEGA) and 2 μg of total RNA were prepared for reverse-transcription reactions. Expression of all four kisspeptin system genes (*kiss1*, *kiss2*, *gpr54-1b* and *gpr54-2b*) was measured in hypothalamus as well as *gnrh-I* by using

qRT-PCR. The mRNA expression levels of *gnrhr-II-1a* and both *fshβ* and *lhβ* were measured in the pituitary. The elongation factor-*1α* (*ef1α*) gene was used as control gene and data were expressed as relative mRNA levels. GSI and gene expression levels (represented as the mean ± sem) were analyzed by a one-way ANOVA followed by the Holm-Sidak test after arcsine and ln-transformation, respectively.

Results and Discussion

Changes in mRNA levels of *kiss1*, *kiss2* and their receptors in the hypothalamus of male sea bass did not follow the changes in the GSI (Table 1). While *kiss1* did not vary significantly among the different testicular stages, *kiss2* expression showed a significant increase in stage I (immature gonads; August) and III (testis in spermatocytic or meiotic phase; October) as compared to testes in stages II (spermatogonial or proliferative phase; September), IV-V (spermiogenic phase; February) or VI (post-spermiation phase; April). The expression of *gpr54-1b* showed a significant elevation in stage III, which was maintained through stages IV-V until the end of the cycle (VI). In contrast, *gpr54-2b* expression displayed significant increases during stages I and V as compared to testes in stages II-IV and the period of resting (VI). On the other hand, changes in gene expression of *gnrhr-II-1a*, but not *gnrh-I*, and both gonadotropins were correlated with the GSI (Table 1). These findings agree with a previous study considering whole brains of male sea bass stocks reared in British waters (6).

Table 1. Kisspeptin and gnrh systems and gonadotropin expression in relation to GSI in European sea bass males.

		Immature/ Early (I/II)	Intermediate (III)	Advanced (IV/V)	Resting (VI)
Hypothalamus	*kiss1*	+	+	+	+
	kiss2	+++/+	+++	+	+
	gpr54-1b	++	+++	+++	+++
	gpr54-2b	+++/+	+	+/++	+
	gnrh-I	+	+	+	+
Pituitary	*gnrhr-II-1a*	+	++	+++	++
	lhβ	++/+	++	++/+++	++
	fshβ	+	++	++/+++	+
Gonad	GSI	+	++	+++	++

Relative gene expression or GSI values: +++ High; ++ Moderate and + Low.

In females, changes in mRNA levels of *kiss1* and *kiss2* and their kisspeptin receptors followed the changes in the GSI (Table 2). The levels of *kiss1* were low-moderate during previtellogenesis (August) and vitellogenesis (November), peaking during the maturation-ovulation stage (February) to then decrease during atresia (April). The first significant increase of *kiss2* occurred during vitellogenesis and displayed maximum levels during maturation-ovulation, showing low values in atresia. The expression of the two kisspeptin receptors was low in the previtellogenesis stage and moderate during the vitellogenesis and maturation-ovulation stages. Low expression levels of both receptors were also observed during atre-

sia. In contrast to the situation in males, changes in all four kisspeptin system genes in females were correlated with changes in *gnrh-I*, *gnrhr-II-1a* and both gonadotropins (Table 2). These results contrast with those found for female Senegalese sole (7), where the expression profile of *kiss2*/*gpr54-2b* in the forebrain did not appear to be correlated with the onset of reproduction (the maturation or the spawing season) of the animals. Results in our study provide evidence of sex differences in the dynamics of expression for two *kiss*/*gpr54* pairs in the hypothalamus of adult male and female sea bass during their first reproductive cycle. These data are in line with the observations in the chub mackerel (8), where a differential expresssion of *kiss* genes was observed in the brain of males and females. While in the grass puffer (9), the expression of *kiss2*/*gpr54-2b* pair was significantly elevated during the spawining season in the brain of both sexes.

Table 2. Kisspeptin and gnrh systems and gonadotropin expression in relation to GSI in European sea bass females.

		Prevtg.	Vtg.	Mat-Ovul.	Atre.
Hypothalamus	*kiss1*	+	++	+++	+
	kiss2	+	++	+++	+
	gpr54-1b	+	++	++	+
	gpr54-2b	+	++	++	+
	gnrh-I	+	++	+++	++
Pituitary	*gnrhr-II-1a*	+	+++	+++	+
	lhβ	+	+++	+++	++
	fshβ	+	+++	+++	+
Gonad	GSI	+	+	+++	+

Relative gene expression or GSI values: +++ High; ++ Moderate; + Low. Prevtg.=previtellogenesis; Vtg.=vitellogenesis; Mat-Ovul.=maturation-ovulation; Atre.=atresia.

The quantification of all four kisspeptin system genes in the pituitary of adult male and female sea bass during different gonadal stages (5) has revealed that their expression levels were lower than those in the hypothalamus. Interestingly, the presence of *kiss* genes in the pituitary suggests that these neuropeptides might have a key role in the regulation of pituitary hormone secretion as well as exhibit some autocrine and/or paracrine actions that merit further investigation.

In summary, the present data in the sea bass and other fish species agree with the putative role that kisspeptins might have in reproduction, similar to those reported in mammals. However, further research is necessary to clarify their specific roles and mode of action in teleosts as they exhibit a variety of reproductive strategies.

Acknowledgements

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CLONING AND CHARACTERIZATION OF ARGININE VASOTOCIN AND ISOTOCIN RECEPTORS IN THE GILTHEAD SEA BREAM (*SPARUS AURATA*)

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Gilthead sea bream (*Sparus aurata*) arginine vasotocin receptors (AVTRs) and isotocin receptor (ITR) cDNAs were isolated and characterized by screening a brain cDNA library using specific probes. These were obtained by PCR using degenerated primers, which were designed in the most conserved regions among different teleost species. The sequences obtained clustered with V1a2-type and V2-type AVTRs, and ITR. The three ORFs contain seven hydrophobic transmembrane domains, with an extracellular N-terminal segment and an intracellular cytoplasmic C-terminal domain. Semiquantitative cDNA expression analysis for the 3 receptors demonstrated that they are ubiquitously and differentially expressed in the 26 tissues examined. This work provides new tools for the understanding of the vasotocinergic and isotocinergic systems in marine teleosts.

Introduction

Arginine vasotocin and isotocin participate in a large variety of physiological processes, such as vascular and osmoregulatory actions, reproduction, behavior, metabolism, stress response, or circadian and seasonal biological rhythms (1). The presence of vasotocin and/or isotocin receptors (AVTR and ITR, respectively) in specific tissues and/or organs indicates a role of both nonapeptides in the physiological processes where these organs are involved (2, 3). In the present study, the mRNAs for AVT and IT receptors from *S. aurata* were cloned, and their tissue distribution was also studied to identify the putative targets, in which both nonapeptides are involved to achieve their final physiological actions.

Material and Methods

A set of degenerate primers was designed according to the most highly conserved sequences of the cDNA among different species for AVTR V1-type, AVTR V2-type and ITR. Total RNA was extracted from the hypothalamus using NucleoSpin® RNA II Kit (Macherey-Nagel). PCR products were identified by electrophoresis in an agarose gel and ligated into the pCR4® vector from TOPO TA Cloning® Kit (Invitrogen™). Following sequencing of clones, we confirmed sequence homology of the PCR products with AVT or IT receptors. The partial sequences obtained were used as probes, labeled with α-^{32}PdCTP and used to screen 250,000 pfu from a brain cDNA library made by our Investigation Group in Lambda Zap (Stratagene, Agilent Technologies; discontinued). After that, positive clones were picked up and plasmid DNA prepared in a mini-prep column [GenElute™ Five-Minute Plasmid Miniprep Kit (SIGMA®)] for sequencing the full-length cDNAs. Phylogenetic analysis conducted in MEGA5 software (4) with the Neighbor-Joining analysis (5)

based on amino acid difference (p-distance) and pairwise deletions. Reliability of the tree was assessed by bootstrap (1,000 replicates) (6). Tissue distribution was achieved by semiquantitative cDNA expression in a total of 26 tissues.

Results and Discussion

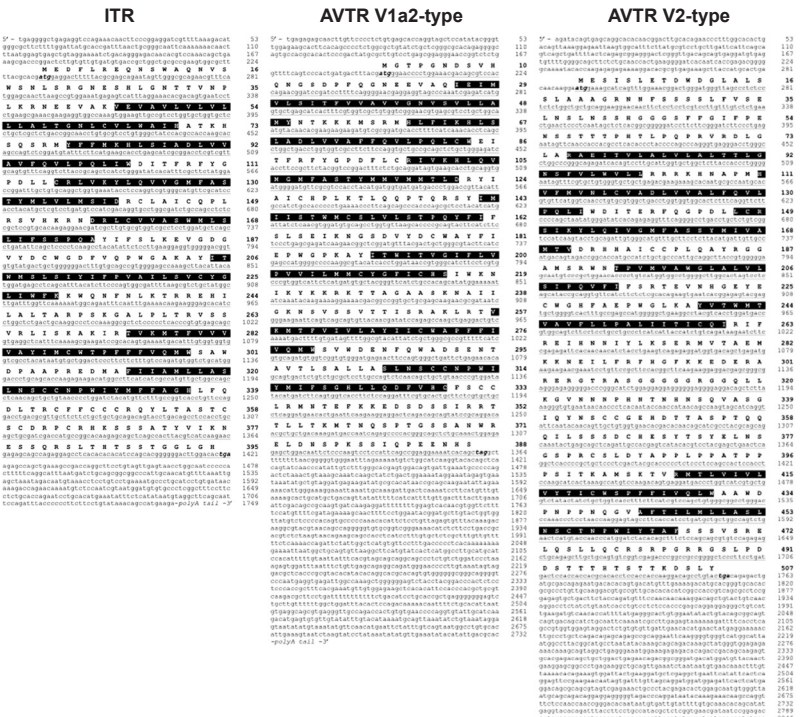

Figure 1. Amino acid sequences of *S. aurata* isotocin (ITR) and vasotocin (AVTR V1a2-type and AVTR V2-type) receptors. The seven transmembrane segments of the open reading frame (ORF) predicted using MEMSTAT (http://bioinf.cs.ucl.ac.uk/psipred/) are represented in bold and highlighted in black in the amino acid sequence.

Nucleotide sequences for the three receptors comprised open reading frames (ORFs) of 1,188 bp, 1,521 bp, and, 1,167 bp encoding for proteins of 395, 506, and 388 amino acids, corresponding to ITR, and V1a2-type and V2-type AVTRs, respectively (Fig. 1), as the longest clone sequences were found to cluster with ITR, and V1a2-type and V2-type AVTRs (Fig. 2). Furthermore, the three ORFs contain seven hydrophobic transmembrane domains, with an extracellular N-terminal segment and an intracellular cytoplasmic C-terminal domain.

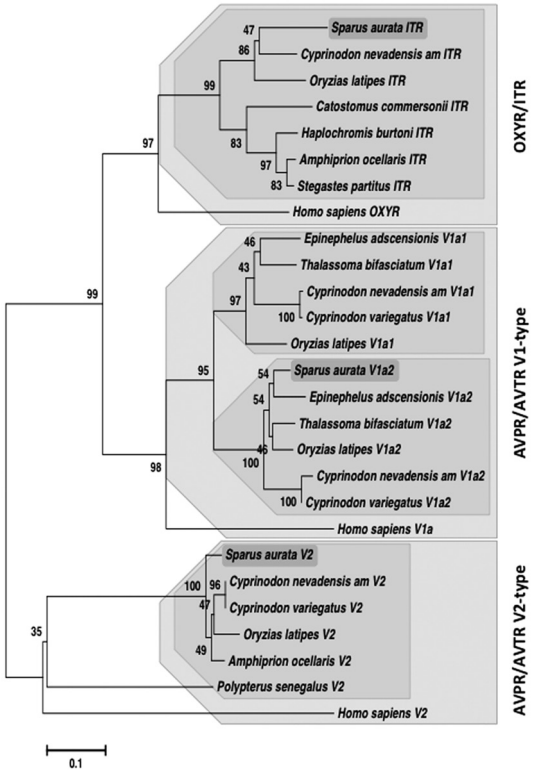

Figure 2. Phylogenetic tree of different vasotocin/isotocin receptors families from several teleosts, including the gilthead sea bream (*S. aurata*), and human using Neighbor-Joining analysis and based on amino acid difference (p-distance).

Semiquantitative cDNA expression analysis for the three receptors demonstrated that they are ubiquitously and differentially expressed in the 26 tissues examined (Fig. 3).

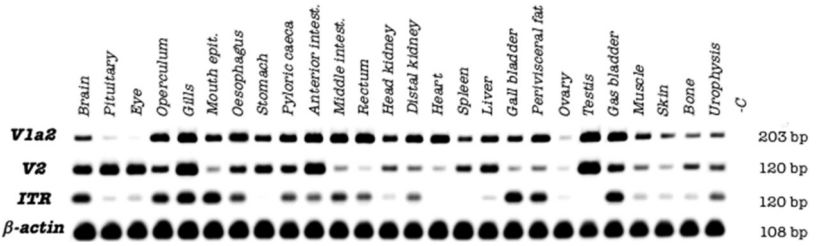

Figure 3. Tissue distribution of AVT receptor V1a2-type, AVT receptor V2-type and IT receptor in *S. aurata* by semiquantitative cDNA expression. PCR products were analyzed by electrophoresis on a 2% agarose gel stained with GelRed. PCR product sizes for each transcript are provided. *–C*: negative control.

AVTR and ITR sequences will be used for the development of methodological tools aimed for a better understanding of their physiological roles in *S. aurata*. In this way and due to the large number of pathways in which those systems are involved, our group is assessing the changes of both systems related to different physiological processes.

Acknowledgements

Study funded by grant AGL2010-14876 (Ministry of Science and Education, Spain) to JMM. JAM-S is supported by a predoctoral fellowship (FPU, Reference AP2008-01194) from Ministry of Education (Spain).

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TISSUE AND SALINITY DEPENDENT EXPRESSION OF VASOTOCIN AND ISOTOCIN RECEPTORS IN MARINE FISH

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A role in osmotic stress has been proposed for the vasotocinergic and isotocinergic systems in different fish species. We assessed, in a time course response study (day 0, 12 hours, and days 1, 3, 7 and 14 post-transfer), the changes in branchial, renal, hypothalamic and hepatic vasotocin and isotocin receptors (AVTRs and ITR, respectively) mRNA expression in specimens of gilthead sea bream (*Sparus aurata*) submitted to acute transfer from 40 ‰ (sea water, SW) to 40 ‰ (control group), 5 ‰ (low salinity water, LSW) and 55 ‰ (high salinity water, HSW). The vasotocinergic system seems to be regulated by salinity and is tissue dependent, with a clear sub-functionalization of each receptor type (V1- or V2-types), with evidences about the putative regulation of different proteins by each one. On the other hand, ITR expression was not disturbed after osmotic challenges. Our results support a clear role for the vasotocinergic, but not the isotocinergic, system in the osmoregulatory processes.

Introduction

The endocrine system plays an important role in the control of the osmoregulatory process, which is responsible for maintaining fish osmotic homeostasis. Several studies in teleosts have pointed out that synthesis of hypothalamic nonapeptides, such as arginine vasotocin (AVT) and isotocin (IT), and their secretion into the blood change in response to environmental salinity (1, 2). The specific receptors of these hormones in several target tissues are the key to produce the physiological action. However, to date, there is only partial knowledge on the functional role of the vasotocinergic and isotocinergic systems throughout its receptors (AVTRs and ITR, respectively) in marine teleosts. In this work, we assessed the mRNA changes in AVTRs and ITR in juvenile specimens of *S. aurata* submitted to different osmotic challenges.

Material and Methods

Immature male specimens of *S. aurata* (80-100 g body weight) were acclimated for 3 weeks in an open circuit of SW (40 ‰ salinity), under constant temperature (18-19 ºC) and natural photoperiod (April-May 2009). After that, specimens were transferred to the three experimental conditions (LSW, SW and HSW) in 400-L tanks with a water recirculation system. Specimens were sampled at day 0, 12 hours, and days 1, 3, 7 and 14 post-transfer. Branchial, renal, hypothalamic and hepatic AVTRs (namely V1a2-type and V2-type) as well as ITR mRNA expression were carried out by total RNA extraction with a commercial kit, cDNA synthesis and qPCR following the $\Delta\Delta C_T$ method (3).

Results and Discussion

In gills, both decrease and increase in environmental salinity up-regulated AVTR V1a2-type expression (Fig. 1.1.a) that was paralleled by Na^+,K^+-ATPase enzyme activity (4); whereas V2-type receptor expression matched with the external salinity (Fig. 1.1.b), as occurred in other transporters like CFTR (5), suggesting a functional association. Moreover, in kidney, V1a2-type AVTR expression peaked at short-term (12 to 24 h) in response to osmotic challenge (Fig. 1.2.a). In contrast, AVTR V2-type had higher expression in the kidney of fish exposed to hyperosmotic environments (SW and HSW) when compared to animals exposed to LSW (Fig. 1.2.b), suggesting a role related to the vasoconstriction function of AVT in the kidney, probably antidiuretic.

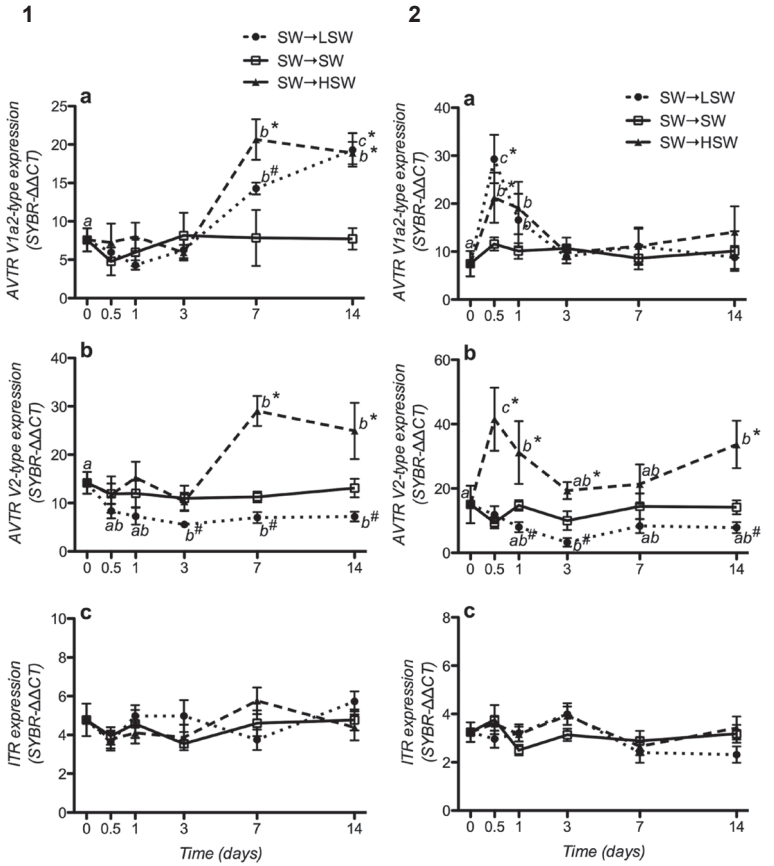

Figure 1. Branchial (1) and renal (2) time course changes in AVTR V1a2-type (a), AVTR V2-type (b) ITR and (c) mRNA expression levels (relative to β-actin) after transfer from SW to different environmental salinities (LSW, SW and HSW). Values are represented as mean ± S.E.M. (n = 8 fish per group). Significant differences within salinity and different time points are identified with different letters; different symbols show differences between groups at the same time (P<0.05, two-way ANOVA followed by Tukey's test).

In the hypothalamus (Fig. 2.1), only V2-type AVTR expression enhanced at both experimental salinities (at 7 and 14 days), suggesting that central control of AVT synthesis and release could be carried out by this AVT receptor. In the liver (Fig. 2.2), both AVTRs increased expression levels, AVTR V2-type up-regulation being faster than V1a2-type. These results suggest that AVTRs are involved in metabolic enzyme regulation, as has been previously reported after AVT treatments (6). Surprisingly, ITR expression was insensitive to variations in external salinity in all tissues analyzed (Figs. 1 & 2). Our results demonstrate the involvement of the vasotocinergic system, but not the isotocinergic, as one modulator of osmoregulatory, metabolic and central controls after osmotic challenge.

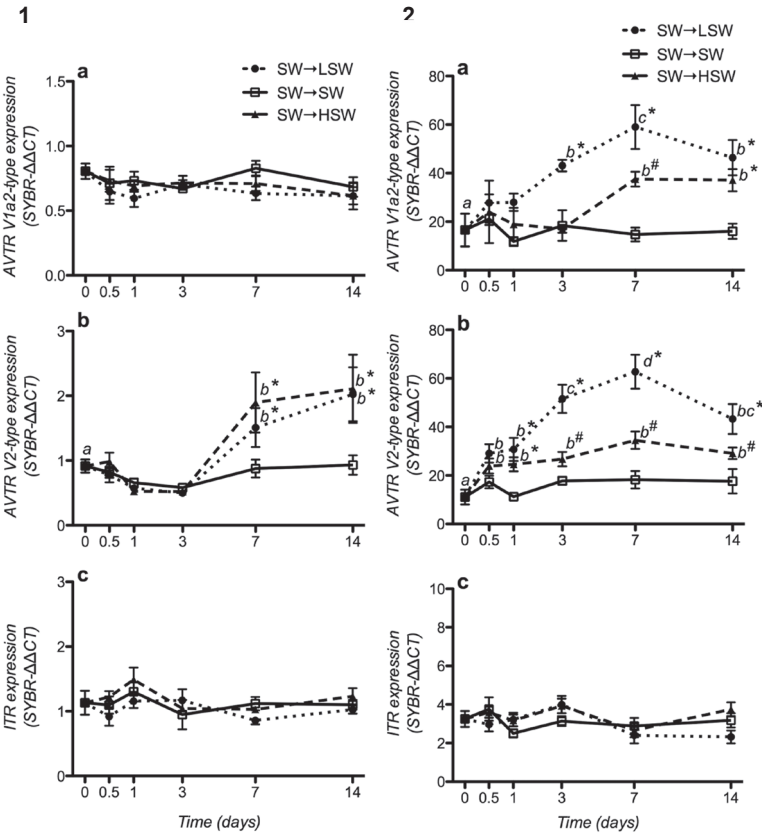

Figure 2. Hypothalamic (1) and hepatic (2) time course changes in AVTR V1a2-type (a), AVTR V2-type (b), and ITR (c) mRNA expression levels. Further details as followed in Figure 1.

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CORTISOL INJECTION MODULATES THE EXPRESSION OF VASOTOCIN AND ISOTOCIN RECEPTORS IN THE GILTHEAD SEA BREAM (*SPARUS AURATA*)

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The present study was designed to investigate the response of arginine vasotocin (AVT) and isotocin (IT) receptors (AVTR and ITR, respectively) in the brain of the gilthead sea bream (*Sparus aurata*) after an intraperitoneal injection with slow release implants of coconut oil alone (control) or containing cortisol (50 $\mu g \cdot g^{-1}$ body weight). Thus, the expression of two AVTRs (namely V1a2- and V2-type) and one ITR were analyzed in both groups in a time-course response (12 hours, and 1, 3, and 10 days post-implantation). Plasma cortisol levels increased in the cortisol-treated group, while no variations in the control group were detected. AVT V1a2-type receptor enhanced its expression after 12 h in the control group, due to the manipulation of the specimens during sham injection handling, while treatment with cortisol down-regulated expression of this receptor. AVTR V2-type expression was stimulated in the cortisol-treated group, suggesting a role of this hormone in metabolic pathways. Finally, ITR presented a delay in its activation as compared with that observed in AVTRs, but it was also sensitive to cortisol treatment. Our results suggest a relationship between vasotocinergic/isotocinergic and stress systems, involving, at least, AVTRs and ITR.

Introduction

In teleosts, the neurohypophyseal hormones arginine vasotocin (AVT) and isotocin (IT) are structurally and functionally similar to arginine vasopressin (AVP) and oxytocin (OXY) in mammals. These neuropeptides are involved in various physiological processes, and their action is determined by their binding to specific receptors in a large variety of tissues. However, to date, there is only partial knowledge about the functional role of AVT and IT in marine fishes after an induced stress situation (1). In teleosts, cortisol is a pleiotropic hormone that presents different physiological roles related to osmotic and ionic regulation, growth and metabolism, as well as stress. In this study, we assessed the effects of cortisol injection on the mRNA expressions of AVT and IT receptors (AVTR and ITR, respectively) in the hypothalamus of the gilthead sea bream (*S. aurata*) along a time course response study.

Material and Methods

Immature male specimens of *S. aurata* (80-100 g body weight) were acclimated in an open circuit of sea water (40 ‰ salinity), under constant temperature (18-19 ºC) and natural photoperiod for our latitude (Puerto Real, Cádiz, Spain, 36° 31’

44'' N). Specimens from the control group were injected intraperitoneally with slow release implants of coconut oil alone, while the experimental group received coconut oil implants containing cortisol (50 µg·g^{-1} body weight). Both groups were sampled at 12 hours, and at 1, 3 and 10 days post injection. Plasma cortisol levels were measured using the ELISA technique. Moreover, the study of the expression levels from hypothalamic AVTRs and ITR was carried out by total RNA extraction, cDNA synthesis and qPCR, using commercial kits.

Results and Discussion

Plasma cortisol levels increased in the cortisol-treated group, while no variations were detected in the control group, demonstrating the effectiveness of the hormonal treatment to enhance plasma cortisol values (Fig. 1).

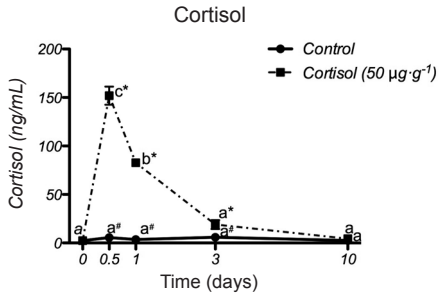

Figure 1. Plasma cortisol levels in *S. aurata* specimens treated with slow release implants of coconut oil alone or containing cortisol (50 µg·g^{-1} body weight).

After 12 h, AVTR V1a2-type expression enhanced in the control group, which was related to the acute stress processes due to the manipulation of specimens during the injection treatment. Moreover, specimens receiving cortisol implants showed a down-regulation process on mRNA expression of this receptor from 12 h until 3 days post-injection (Fig. 2a). These results suggest a functional association between cortisol release and the AVT V1a2-type receptor. AVTR V2-type expression levels were only stimulated in the cortisol-treated group from 12 h to 3 days post-injection, returning close to control values at day 10 of the experiment. However the control group maintained stable values during the time of the experiment, suggesting that stress situation (cortisol enhancement) activates metabolic pathways regulated *via* AVT V2-type receptor (2) (Fig. 2b).

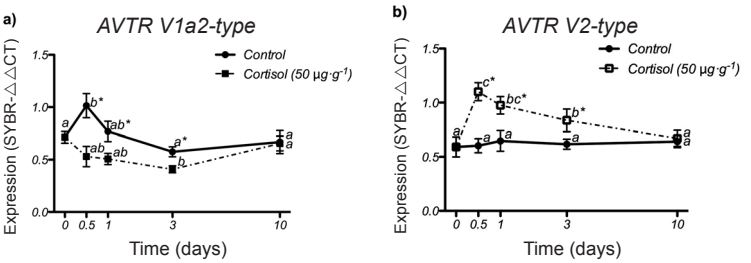

Figure 2. Hypothalamic AVTR V1a2-type (a) and AVTR V2-type (b) mRNA expression changes in specimens of *S. aurata* treated with slow release implants of coconut oil alone (control) or containing cortisol (50 µg·g^{-1} body weight).

ITR expression was also sensitive to cortisol treatment from day 1 till day 3, showing a delay in its activation as compared with the responses in AVTRs. The control group remained without variation throughout the whole time of the experiment (Fig. 3).

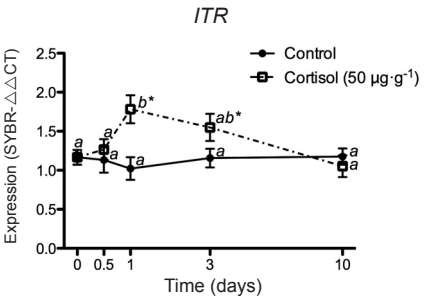

Figure 3. Hypothalamic ITR mRNA expression in specimens of *S. aurata* treated with slow release implants of coconut oil alone (control) or containing cortisol (50 µg·g^{-1} body weight).

These results suggest an interaction between stress axis and vasotocinergic and isotocinergic systems. However, further studies with traditional experiments related to stress axis activation (e.g. air exposure, see 3) are necessary in order to clarify this relationship.

Acknowledgements

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CIRCADIAN EXPRESSION OF MELATONIN RECEPTOR SUBTYPES $MEL_{1a}1.4$ AND $MEL_{1a}1.7$ IN CENTRAL AND PERIPHERAL LOCATIONS IN GOLDFISH (*CARASSIUS AURATUS*)

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Melatonin is considered to act as an important neuroendocrine transducer of environmental information. Four subtypes of melatonin receptors ($Mel_{1a}1.4$, $Mel_{1a}1.7$, Mel_{1b} and Mel_{1c}) are thought to be mediating physiological functions of this hormone in non-mammalian vertebrates. The aim of this study was to characterize possible daily variations in the expression of some of these melatoninergic receptors under different light and feeding conditions using goldfish (*Carassius auratus*) as a teleost model. Fish were acclimated for 15 days under a 12 h of light and 12 h of darkness (12L:12D) photoperiod and fed once daily at ZT2. Then, three experimental groups were established: 12L:12D and scheduled feeding at ZT2 (Group I), 12L:12D and random schedule feeding (Group II), and 24L with scheduled feeding at CT2 (Group III). After two weeks, central and peripheral tissue samples were collected at ZT1, ZT7, ZT13, ZT19 and ZT1b throughout a 24 h cycle. $Mel_{1a}1.4$ and $Mel_{1a}1.7$ gene expression was determined using *real-time* RT-PCR. Results show that both receptor subtypes are expressed in central locations, with significant daily variations found only in the optic tectum for $Mel_{1a}1.4$ under constant light conditions, and in the retina for both receptors in Group I, in all cases maximum levels were detected at ZT/CT13. As for peripheral tissues, $Mel_{1a}1.4$ and $Mel_{1a}1.7$ seem to be synchronously expressed in the foregut with a peak at ZT7 in Group I, which is consistent with low levels of circulating melatonin. In the hindgut and head kidney, neither $Mel_{1a}1.4$ nor $Mel_{1a}1.7$ show a rhythmic expression under a 12L:12D photoperiod and a scheduled feeding at ZT2. These results demonstrate the presence of melatonin receptors and their daily expression not only in central, but also in peripheral tissues of goldfish.

Introduction

Melatonin (MEL, N-acetyl-5-methoxytryptamine) is produced mainly in the pineal gland and retina and is considered to act as an important neuroendocrine transducer of photoperiod. MEL is involved in numerous physiological processes including circadian entrainment, retinal physiology and seasonal reproduction, among others (1). In non-mammalian vertebrates, four subtypes of G protein-coupled receptors ($Mel_{1a}1.4$, $Mel_{1a}1.7$, Mel_{1b} and Mel_{1c}) are thought to be mediating functions of this hormone (2). The aim of this study was to characterize possible daily variations in the expression of some of these melatoninergic receptors under different light and feeding conditions using goldfish as a teleost model.

Materials and Methods

Goldfish (*Carassius auratus*) were acclimated for 15 days under 12 h light and 12 h darkness (12L:12D) photoperiod and fed once daily at *zeitgeber* time 2 (ZT2). Then, three experimental groups were established: 12L:12D and scheduled feeding (SF) at ZT2, 12L:12D and random feeding (RF), and 24L with SF at circadian time 2 (CT2). After two weeks, fish were fasted for 24 h and sacrificed throughout a 24-h cycle at ZT1, ZT7, ZT13, ZT19 and ZT1 of the following day (ZT1b) to collect samples of optic tectum, retina, foregut, hindgut and head kidney. $Mel_{1a}1.4$ and $Mel_{1a}1.7$ gene expression was analyzed by quantitative real time-PCR, using the $2^{-\Delta\Delta Ct}$ method to determine the relative mRNA expression and the primers previously reported (1). Statistical differences among sampling points were determined by one-way ANOVA followed by the SNK test when necessary. The significance of the rhythms was analyzed by fitting data to sinusoidal functions by cosinor analysis.

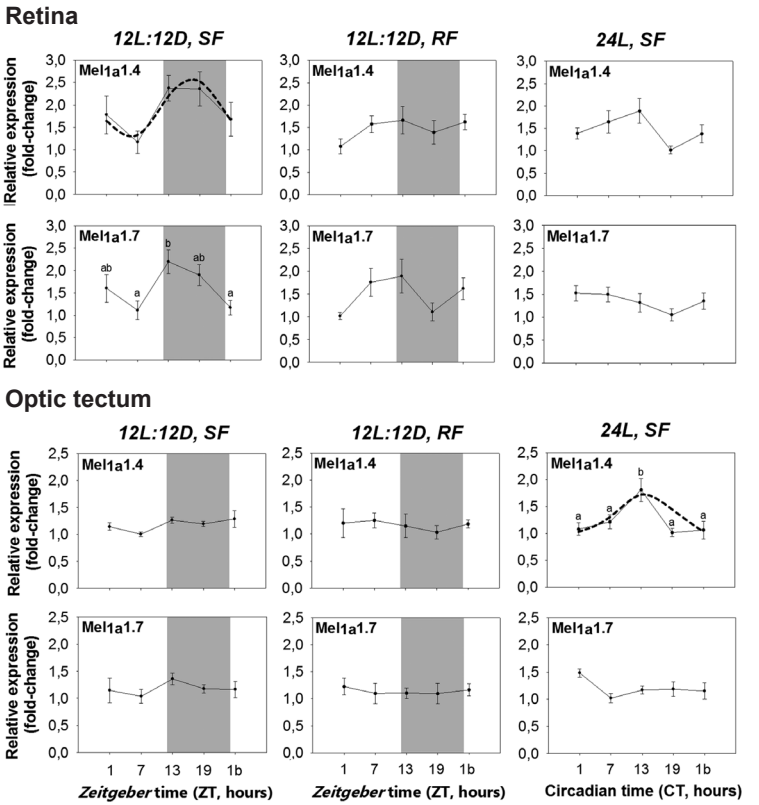

Figure 1. Relative expression of $Mel_{1a}1.4$ and $Mel_{1a}1.7$ in central locations in goldfish. SF, scheduled feeding at ZT/CT-2 and RF, random feeding. The transcript relative amount is expressed as mean ± SEM (n=6). Different letters indicate significant differences by post-hoc analysis ($p<0.05$). Dashed lines represent significant daily rhythms by cosinor analysis. Shaded area indicates the dark period.

Results and Discussion

1. Expression of $Mel_{1a}1.4$ and $Mel_{1a}1.7$ in central locations

Both $Mel_{1a}1.4$ and $Mel_{1a}1.7$ are expressed in central locations (Fig. 1), in accordance with previous studies in goldfish (1). In the retina, expression of both receptors showed significant daily variations under 12L:12D and SF at ZT2, while no significant changes were found in the other two conditions tested. In the optic tectum, no significant rhythmic variations were observed, except for $Mel_{1a}1.4$ under 24L. These results indicate that both *zeitgebers*, light-dark cycle and scheduled feeding seem to be important for synchronization of melatonin receptor expression in the retina, although the role of a scheduled feeding in the optic tectum needs to be clarified.

2. Expression of $Mel_{1a}1.4$ and $Mel_{1a}1.7$ in peripheral locations

Daily expression of $Mel_{1a}1.4$ and $Mel_{1a}1.7$ in peripheral locations is shown in Fig. 2. In the foregut, both receptors showed similar daily profiles with a peak at ZT7, when circulating melatonin levels are low (3). These variations were only observed under 12L:12D photoperiod and SF at ZT2, suggesting again the relevance of both *zeitgebers* for rhythmicity of melatonin receptors expression. As for the hindgut and head kidney, neither $Mel_{1a}1.4$ nor $Mel_{1a}1.7$ showed a rhythmic expression under 12L:12D photoperiod and SF (data not shown). These results demonstrate for the first time the presence of melatonin receptors in the gut and head kidney in goldfish, in consistence with the various peripheral actions of this hormone (4).

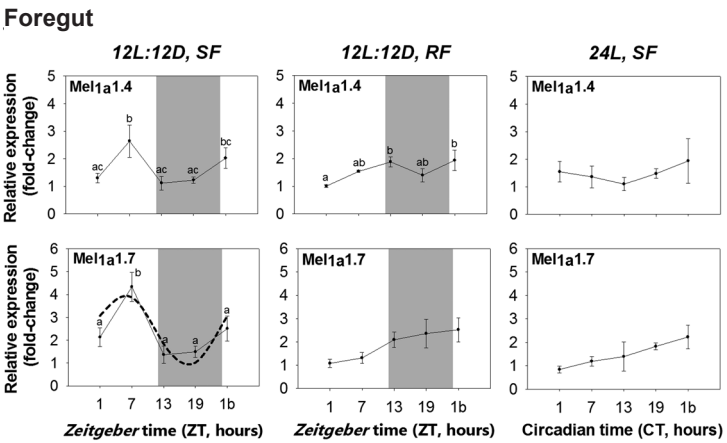

Figure 2. Relative expression of $Mel_{1a}1.4$ and $Mel_{1a}1.7$ in peripheral locations in goldfish. SF, scheduled feeding at ZT/CT-2 and RF, random feeding. The transcript relative amount is expressed as mean ± SEM (n=6). Different letters indicate significant differences by post-hoc analysis ($p<0.05$). Dashed lines represent significant daily rhythms by cosinor analysis. Shaded area indicates the dark period.

Acknowledgements

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TIME-COURSE OF THE LIVER METABOLIC RESPONSE TO ACUTE STRESS IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

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Stress jeopardizes physiological homeostasis in all studied animals. Negative effects of stress have been shown to affect a number functions in fish (food intake, reproduction, growth and immune system), but the response of several metabolism-related parameters in the liver has not been elucidated. This study evaluates the effect of acute stress on the activity and expression of metabolic enzymes in the liver of rainbow trout. Animals were either subjected to acute stress (5 minutes of repeated chasing) or not (controls), and sacrificed at 15, 45, 120, 240, and 480 minutes post-stress. In general, parameters related to glucose metabolism were significantly affected by acute stress, but most of them recovered to normal values after 480 minutes. GK, PEPCK and PK activities were inhibited by acute stress, but 480 minutes was not time enough for G6Pase increased activity to recover. Significantly inhibited expression of *GK*, *PEPCK* and *GLUT2* genes was also noted, whereas that of *G6Pase* and *PK* was up-regulated. Such effects dissipated after 480 min. Lipid metabolism in the liver was also affected by acute stress. FAS activity and mRNA abundance were significantly inhibited, while HOAD activity increased at 45 minutes post-stress. These variations also disappeared after 480 minutes. Detailed evidence showing that acute stress is temporally jeopardizing the typical profile of liver metabolism, affecting those parameters related to glucose and lipid metabolism is reported. In conclusion, our results demonstrate the impact of acute stress on liver function, which includes molecular reprogramming essential for fulfilling all the energy requirements enhanced by stress exposure.

Introduction

Stress negatively affects several functions in fish (food intake, reproduction, growth, and immune functions) (1), but also induces tissue specific metabolic reprogramming to cope with the increased energy demands associated with stress, especially in tissues like the liver, which is crucial to stress adaptation (1). This metabolic reprogramming in liver tissue is related in a first stage to the action of enhanced circulating levels of catecholamines, while in a second stage metabolic reallocation is basically dependent on cortisol action (2). Handling of fish is a common and unavoidable procedure in fisheries management and the aquaculture industry that induces short-term acute stress effects. Since the physiological response to acute stressors, like handling, has been evaluated in fish of interest in aquaculture, such as the rainbow trout, only a few parameters have been assessed. Therefore, the present study describes the short-term time-course of metabolic response in the liver of rainbow trout after acute handling stress, by evaluating enzyme activity and mRNA abundance in parameters related to carbohydrate and lipid metabolism.

Materials and Methods

Two cohorts of rainbow trout (93 ± 7 g; N = 48 each) were distributed in 120l tanks (n = 8/tank) adapted to laboratory conditions. On the day of sacrifice, fish were sampled from both cohorts as 0 time groups. The remaining fish from the first cohort, the handling group, were exposed to acute handling disturbance (chasing for 5 min), while animals from the other cohort, the non-stressed control group, remained unaltered. Fish from each cohort were deeply anaesthetized (0.2% of 2-phenoxyethanol) at different post-stress periods (0 and 15, 45, 120, 240, and 480 min) and the livers were immediately sampled, frozen on dry ice and stored at -80ºC until assessed for enzyme activity (GK, PEPCK, G6Pase, PK, GPase, GSase, FAS and HOAD) and gene expression (*GK*, *PEPCK*, *G6Pase*, *PK*, *GLUT2* and *FAS*).

Results and Discussion

The activity and gene expression of enzymes related to glucose and lipid metabolism is shown in Figure 1. Stress negatively affected the activity of GK, PEPCK and PK, but stimulated G6Pase activity at 45 min post-stress. No changes were noted for GPase and GSase activity, but their ratio increased in stressed fish at 15 min post-stress relative to controls. *GK*, *PEPCK* and *GLUT2* expression significantly decreased in stressed fish relative to controls, whereas that of *G6Pase* and *PK* increased. 480 minutes was enough time for all gene measurements to return to those of non-stressed control group fish.

Regarding lipid metabolism, FAS activity in stressed fish decreased, reaching a minimum at 45 min post-stress. FAS expression in control fish decreased after 240 and 480 min whereas in stressed fish such decline occurred from 45 to 240 min, with levels being lower than those of controls. HOAD activity in stressed fish was higher than that of controls at 45 min post-stress.

Understanding the nature of the physiological response of farmed fish to aquaculture-related stressors is necessary for improving animal welfare. Accordingly, we report the short-term time course of the liver metabolic response to acute handling stress in rainbow trout. The present results support the existence of two stages in the time-course of metabolic response. A first stage occurring some minutes post-stress (15-45 min) is characterized by increased mobilization of liver glycogen resulting in increased production of endogenous glucose, reduced use of exogenous glucose and reduced lipogenic potential. The second stage (60-120 min onwards) is characterized by the recovery of liver glycogen levels, increased capacity of the liver for releasing glucose and the recovery of lipogenic capacity, while no changes were noted in gluconeogenic capacity, which probably needs longer time periods to be enhanced. Those metabolic stages may associate with the differential temporal changes in plasma hormone levels occurring after stress, i.e. the fast and short-term rise in catecholamine levels (2) and increased cortisol levels (1, 2, 3, 4).

Acknowledgements

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0000 300S 14008) and Ángeles Alvariño programmes (Xunta de Galicia). Librán-Pérez is recipient of a FPI fellowship.

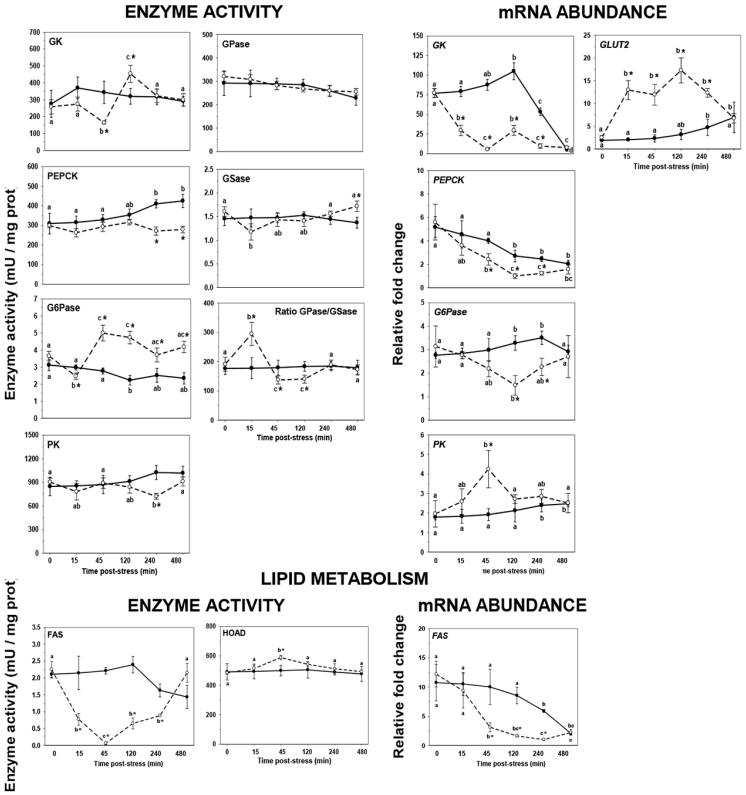

Figure 1. Time course of activities and mRNA abundance in parameters related to glucose and lipid metabolism in liver of trout acutely stressed by 5 min handling (white symbols and dashed line) or not (black symbols and solid line) and sampled at 0 to 480 min post stress. Data represent the average ± S.E.M. of 8 fish. * Significantly different (P<0.05) than control non-stressed group at the same time. Different letters indicate significant differences (P<0.05) among sampling times within the same treatment.

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SIGNALING PATHWAYS ACTIVATED BY β_2-ADRENOCEPTOR AGONISTS IN GILTHEAD SEA BREAM (*SPARUS AURATA*) CULTURED MYOCYTES

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Activation of β_2-adrenergic receptors increases protein synthesis and reduces degradation, causing muscle hypertrophy. The Gα subunit activates cAMP production, which induces myocyte proliferation and differentiation and the expression of important myogenic regulatory factors. Moreover, Gβγ activates the PI3K/AKT/mTOR signaling pathway, involved among other functions in protein synthesis. We used primary cultured myocytes as a model to study the activation of signaling pathways dependent on β_2-adrenoceptors in gilthead sea bream. Specifically, we incubated day 4 myocytes with noradrenaline or formoterol at 1 μM. We evaluated cAMP levels as well as AKT and mTOR phosphorylation. Results have shown that sea bream myocytes are quite sensitive to β-adrenergic stimulation. cAMP levels were increased in response to both agonists, noradrenaline and formoterol. Nevertheless, the delayed activation observed with formoterol in comparison to noradrenaline suggested longer-lasting effects for the former. Moreover, increased mTOR phosphorylation was also detected in response to both treatments, indicating a possible induction of protein synthesis. However, neither noradrenaline nor formoterol caused a clear effect on AKT activation. Furthermore, the possible effect of β_2-agonists on the cells' proliferative capacity, by means of Proliferating Cell Nuclear Antigen expression, was analyzed by western blot in the same samples. Nevertheless, differences after treatments were not observed, thus suggesting that longer incubations might be required to stimulate proliferation in these cells. Overall, the present study has demonstrated that the signaling pathways activated by β-adrenergic agonists are conserved in fish myocytes. Nevertheless, further studies are necessary to unravel the effects of β-adrenergic activation in fish muscle growth and development.

Introduction

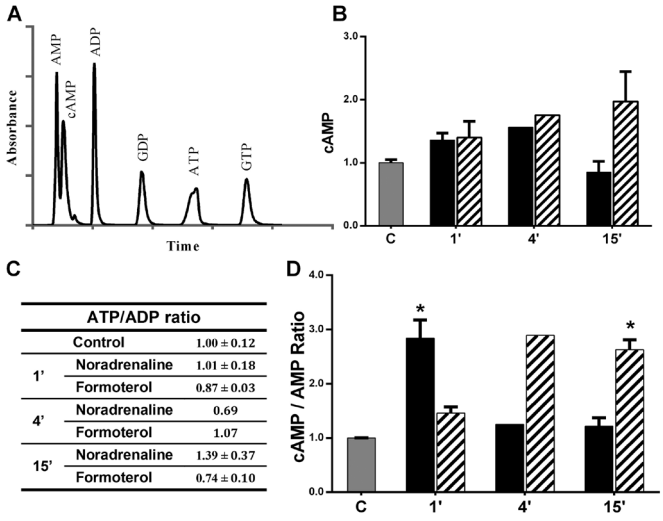
β-adrenoceptor signaling plays an important role in skeletal muscle growth and development. Stimulation of β_2-adrenergic receptors increases protein synthesis and decreases degradation, leading to muscle hypertrophy. Agonist-receptor association causes G-protein dissociation into two parts. Gα activates cAMP production, which in turn activates protein kinase A (PKA). In mammals, myocyte PKA signaling pathway activation induces proliferation and differentiation, as well as the gene expression of important myogenic regulatory factors like Pax3, MyoD and Myf5 (1). On the other hand, Gβγ activates myocyte PI3K/AKT/mTOR signaling pathway, involved in protein synthesis, cell proliferation and survival (2). The aim of the present work was to study the signaling pathways dependent on the activation of β_2-adrenoceptors in gilthead sea bream muscle cells and to evaluate the possible effect of β_2-agonists on the cells' proliferative capacity.

Materials and Methods

Primary cultures of gilthead sea bream muscle cells were performed following the procedure previously described by Montserrat et al. (3). Myocytes at day 4 were starved for 2 h with DMEM containing 0.02% fetal bovine serum and 1% antibiotic/antimycotic solution. Cells were then treated with noradrenaline or formoterol (a β_2-agonist) at 1 μM concentration each or left untreated (C), and samples were taken at different times. To evaluate Gα activation, soluble cellular fraction was obtained by an acid extraction (10% trichloroacetic acid) and analyzed by an HPLC method adapted from Casals et al. (4). To measure cAMP levels, the specific peak's area was quantified using the GRAMS *Suite* 9.1 software. Next, Gβγ activation was evaluated through the analysis of the PI3K/AKT/mTOR signaling pathway by western blot. Cell protein lysates were extracted with RIPA buffer, and then AKT and mTOR phosphorylation and Proliferating Cell Nuclear Antigen (PCNA) protein expression was determined.

Results and Discussion

1. cAMP levels. Fig. 1A shows a representative HPLC profile of cellular adenylic components. In gilthead sea bream, myocytes incubated with both, noradrenaline or formoterol, cAMP levels increased in a few minutes (Fig. 1B), without altering the cellular metabolic status as indicated by the stable ATP/ADP ratios observed (Fig. 1C). Moreover, the ratio cAMP/AMP evidenced the significant increase of cAMP and the rapid hydrolysis to AMP in these cells, which was very quick after noradrenaline stimulation, but delayed in response to formoterol (Fig. 1D). These results indicate a possible longer acting effect in muscle of formoterol in comparison with noradrenaline.



ATP/ADP ratio		
	Control	1.00 ± 0.12
1'	Noradrenaline	1.01 ± 0.18
	Formoterol	0.87 ± 0.03
4'	Noradrenaline	0.69
	Formoterol	1.07
15'	Noradrenaline	1.39 ± 0.37
	Formoterol	0.74 ± 0.10

Figure 1. HPLC results from the β-adrenergic stimulation of gilthead sea bream myocytes at day 4 incubated with either ■ noradrenaline or ▨ formoterol at 1 μM for 1, 4 or 15 min or left □ untreated (C). **A.** Example of a chromatographic profile of different commercial nucleotides. **B.** cAMP levels. **C.** ATP/ADP ratio **D.** cAMP/AMP ratio. Results are presented as fold change over control. Mean ± SEM (n=2). Significant differences were considered at $p<0.05$.

2. Western blot analyses. Results show that both agonists significantly increased the phosphorylation of mTOR at 15 and 60 min compared to control cells (Fig. 4A), suggesting a possible stimulation on protein synthesis, whereas AKT remained unaltered (Fig. 4B). Furthermore, differences in PCNA expression were not observed upon treatment; thus suggesting that long-term incubations might be required to stimulate myocyte proliferation (Fig. 4C).

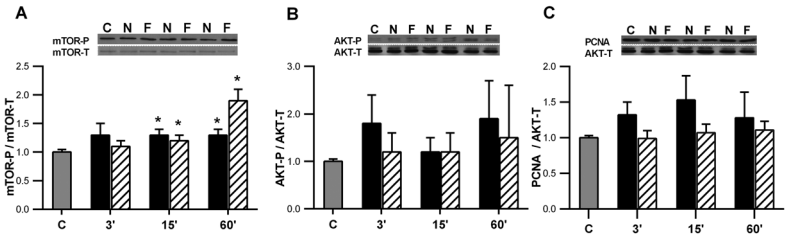


Figure 2. Western blot analyses of gilthead sea bream myocytes at day 4 incubated with either ■ noradrenaline or ▨ formoterol at 1 *μ*M at 3, 15 and 60 min or left ▩ untreated (C). Representative images and quantification of **A.** mTOR, **B.** AKT and **C.** PCNA western blots. Phosphorylated mTOR (mTOR-P) and AKT (AKT-P) were normalized in relation to their corresponding total form (mTOR-T and AKT-T, respectively), and PCNA *versus total* AKT (AKT-T). Results are presented as fold charge over control. Mean ± SEM (n=2). Significant differences were considered at $p<0.05$.

Overall, these results have indicated that gilthead sea bream myocytes are quite sensitive to β-adrenergic stimulation and suggest that the signaling pathways activated through both Gα and Gβγ subunits are well-conserved among fish and mammals. Nevertheless, further studies also *in vivo* are necessary to discover whether β-adrenergic activation in fish muscle may have a role in hypertrophy.

Acknowledgements

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EFFECTS OF OLEOYLETHANOLAMIDE (OEA) ON FOOD INTAKE AND LOCOMOTOR ACTIVITY IN GOLDFISH (*CARASSIUS AURATUS*)

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Energy homeostasis in vertebrates is regulated by neuroendocrine systems by adjusting caloric intake to energy expenditure. Oleoylethanolamide (OEA), an endogenous lipid synthesized in the intestine, plays a significant role in the regulation of energy balance in mammals, modulating feeding, locomotor activity and lipid metabolism. To date, the role of OEA in non-mammalian vertebrates and particularly in fish remains to be explored. The aim of the present study was to elucidate the possible role of OEA on food intake and locomotor activity in the cyprinid *Carassius auratus*. Goldfish were intraperitoneally (IP) injected with 10 µl vehicle/g body weight (bw) or containing OEA (5 µg/g bw). Food intake was individually quantified at different time intervals post-injection (0-2, 2-8 and 0-8 h). Swimming activity was registered in tanks with 6 fish at the same time periods. Blood samples were collected at 2 h post-injection to quantify plasma glucose and triglyceride levels. The IP administration of OEA significantly reduced food intake and swimming activity at 2 h post-injection, but not during the next discrete interval (2-8 h), suggesting that OEA acts at short time in goldfish, in agreement with previous findings in mammals. A significant reduction in circulating triglycerides induced by OEA was observed at 2 h post-injection, as reported in mammals. In summary, present results show for the first time in a non-mammalian vertebrate that OEA, a bioactive lipid amide, may be involved in the regulation of feeding, locomotor activity and lipid metabolism.

Introduction

Feeding behavior in vertebrates is a process regulated by central and peripheral signals including several neurotransmitters, hormones, peptides and metabolic intermediates. The gastrointestinal tract plays an essential role in the regulation of feeding by both neuronal and humoral mechanisms. OEA is a fatty acid ethanolamide mainly produced in the small intestine from food-derived oleic acid (1) and involved in feeding, locomotor activity and lipid metabolism regulation in rodents (2-5). The physiological actions of OEA have not yet been investigated in non-mammalian vertebrates. In the present study, we investigate the possible role of OEA in food intake and locomotor activity in the cyprinid goldfish (*Carassius auratus*).

Materials and Methods

Goldfish were intraperitoneally (IP) injected at feeding time (10:00) with 10 µl of vehicle alone (5% Tween 20, 5% polyethylene glycol, 90% teleost saline) per g bw or containing OEA (5 µg/g bw). Food intake was individually quantified in goldfish (16 fish/group) maintained in 5-l aquaria at 0-2, 2-8 and 0-8 h post-injection.

Swimming activity was registered in groups of 6 fish held in 60-l tanks (6 tanks/group) following the protocol used by Azpeleta et al. (6) at the same time intervals as food intake quantification. Swimming activity was expressed as a percentage in relation to the previous activity registered in each tank the day prior to the treatment at the same time period. Blood samples were collected at 2 h post-injection under two feeding conditions, with and without food after IP injections. Glucose and triglyceride plasma levels were quantified using an enzymatic/colorimetric method with commercial kits. Results were analyzed by Student's t-test in order to ascertain statistical differences between control and OEA treated fish.

Results and Discussion

1. Effect of OEA on food intake. The OEA IP treatment (5 µg/g bw) significantly reduced food intake at 2 and 8 h post-injection (Fig. 1A), which decreased around 70% at 2 h. The fact that the feeding decrease due to OEA was observed during the first 2 h after injection, but not during the next discrete interval (2-8 h) may indicate that this ethanolamide acts at short time in goldfish. This result suggests that OEA may to be involved in feeding regulation in goldfish, as previously described in mammals (2,4).

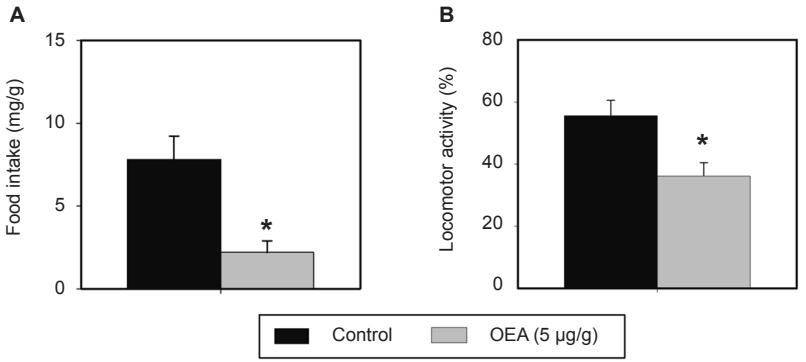

Figure 1. Food intake (A) and locomotor activity (B) at 2 h after acute IP administration of vehicle or OEA (5 µg/g) in goldfish. Data are expressed as mean ± SEM (*: $p < 0.05$).

2. Effect of OEA on swimming activity. The IP administration of OEA (5 µg/g bw) significantly decreased activity at 2 h post-injection (Fig. 1B) in goldfish, but did not modify the swimming activity at 2-6 h and 0-8 h intervals. The total activity during the light and dark phases of the daily photocycle was not modified by an acute OEA injection. As previously reported, the OEA induced anorexigenic effect in mammals is accompanied by a reduction in locomotor activity at short time (3, 4). The fact that both OEA induced actions appear at the same time period could suggest some interactions between them. The inhibitory effect of OEA on food intake might be a consequence of the reduction of swimming activity; and alternately the anorectic action of OEA could be the ultimate factor that reduces swimming activity. However, previous data in rats do not support such interactions (4).

3. Effect of OEA on plasma metabolites. Glycemia was not significantly modified by the acute OEA treatment. Plasma triglyceride levels were significantly reduced at 2 h after OEA IP treatment (5 µg/g) compared to the control fish in both experimental groups (control: 2.40 ± 0.16, OEA: 1.30 ± 0.11 mg/ml in fed fish; control: 1.86 ± 0.28, OEA: 1.30 ± 0.11 mg/ml in 26 h-fasted fish). This reduction in plasma triglycerides agrees with the reported lipolytic action of OEA in mammals (5), although more studies are needed to elucidate the role of OEA in lipid metabolism in fish.

In conclusion, these results suggest that OEA, a fatty acid ethanolamide, seems to be involved in locomotor activity and feeding regulation in fish, and point to an evolutionary conservation of OEA actions in vertebrates.

Acknowledgements

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EFFECTS OF L-LEUCINE, L-ARGININE AND GROWTH HORMONE ON LIPOLYSIS IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) ADIPOCYTES

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In order to investigate how growth and nutritional factors are involved in lipid hydrolysis and storage in fish, we examined the effects of two amino acids (L-leucine and L-arginine) at two different concentrations, and of the growth hormone (GH) and insulin-like growth factor-I (IGF-I) also at different doses, on lipid mobilization and gene-related expression in freshly isolated adipocytes from rainbow trout (*Oncorhynchus mykiss*). Lipolysis was measured as the amount of glycerol released into the medium. GH induced a significant increase in glycerol levels in a dose-response manner, while IGF-I inhibited lipolysis. Moreover, GH did not change IGF expression, thus indicating a direct lipolytic effect of GH. Lipolysis measurements were significantly higher in isolated adipocytes incubated with high doses of L-arginine (6 mM) and L-leucine (4 mM), whereas low doses did not show differences in comparison with the control condition. In summary, the present study contributes to improving knowledge on the regulatory role of GH and amino acids in trout adipocyte metabolism to further optimize feed efficiency and diet composition.

Introduction

Adipose tissue is one of the major energy storage sites for fat. Lipid content is determined by the balance between lipid mobilization (lipolysis) and deposition according to the animal's requirements (1). It has been demonstrated that the growth hormone (GH) plays an important role in fish lipolysis (2), but the GH's direct or indirect actions (mediated by insulin-like growth factors or IGFs) in adipose tissue remain unknown. Previous experiments have shown that diet composition, especially the amino acid profile, also has an important effect on lipid mobilization from adipose tissue in teleosts (3).

Materials and Methods

Adipocytes were isolated from visceral adipose tissue of rainbow trout (*Oncorhynchus mykiss*). Fat tissue was cut into thin pieces and incubated in a shaking water bath at 18 °C for 60 min with Krebs-Hepes buffer (pH 7.4) containing collagenase type II (130 U ml^{-1}) and 1% bovine serum albumin (BSA). The cell suspension was filtered through a cell strainer (100 µm) and then washed twice by flotation. Finally, cells were carefully resuspended at the desired concentration in Krebs-Hepes buffer containing 2% BSA. Triplicates of 1 ml of the final adipocyte suspension were incubated in polypropylene tubes for 3 h at 18 °C in the absence (control)

or presence of L-leucine (2 and 4 mM), L-arginine (4 and 6 mM), GH (0,1, 1 and 10 nM) or IGF-I (10 and 100 nM). At the end of the incubation time, the cells were centrifuged at 2700 *g* for 2 min at 4 °C. Six hundred µl of medium were placed in new tubes for glycerol analysis with the Serum Triglyceride Determination Kit (Sigma-Aldrich, Tres Cantos, Spain), and the cells from each triplicate were recovered together in 1 ml of TRIreagent (Ambion, Alcobendas, Spain) for RNA extraction, cDNA synthesis and subsequent gene expression analyses by quantitative real-time PCR.

Results and Discussion

1. Hormone effects on lipolysis. GH induced a significant increase in glycerol levels in the media at all three different doses tested, whereas IGF-I incubation inhibited lipolysis (Fig. 1). Thus, we can state that GH has a lipolytic effect in rainbow trout isolated adipocytes as previously described in gilthead sea bream (2) and, for the first time in fish, that IGF-I has an antilipolytic effect in this cell model.

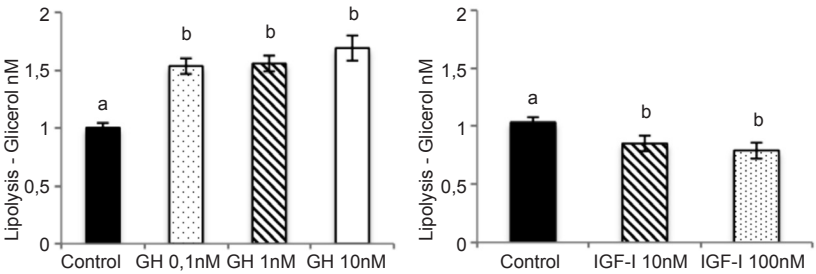

Figure 1. Effects of incubations with GH (A) or IGF-I (B) on lipolysis in isolated rainbow trout (*Oncorhynchus mykiss*) adipocytes. Data are shown as mean values ± SEM. Significant differences between treatments and control condition are shown with different letters (p<0,05).

2. Amino acid effects on lipolysis. The highest doses used of L-arginine (6 mM) and L-leucine (4 mM) induced a significant increase in glycerol release into the media, whereas lower doses did not show differences in comparison with the control condition (Fig. 2). These results suggest that the different composition of amino acids in the diet may affect growth and the mobilization of fat in these animals.

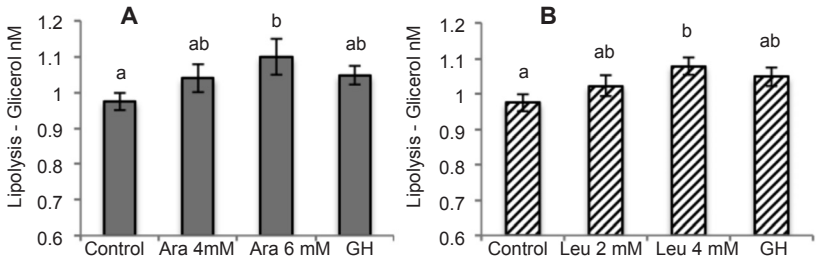

Figure 2. Effects of incubations with GH (10 nM) and two different concentrations of L-arginine (A) and L-leucine (B) on lipolysis in isolated rainbow trout (*Oncorhynchus mykiss*) adipocytes. Data are shown as mean values ± SEM. Significant differences between treatments and control condition are shown with different letters (p<0,05).

3. Hormone effects on gene expression. Quantitative real-time PCR was used to measure expression changes of IGF-I and IGF-II in response to GH incubation. GH did not change IGFs expression (Fig. 3), thus indicating a direct lipolytic effect of GH in rainbow trout adipocytes.

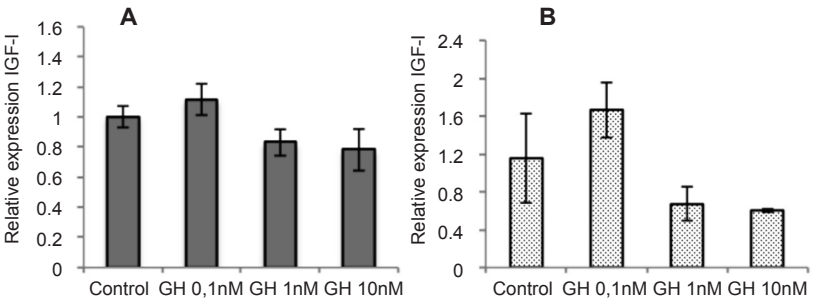

Figure 3. Effects of incubations of three different concentrations of GH on the relative gene expression of IGF-I (A) and IGF-II (B) in isolated rainbow trout (*Oncorhynchus mykiss*) adipocytes. Data are shown as mean values ± SEM. No significant differences were observed ($p<0,05$).

Conclusions

It has been demonstrated that GH acts as a lipolytic hormone, whereas IGF-I presents insulin-like effects, inhibiting lipolysis in isolated rainbow trout (*Oncorhynchus mykiss*) adipocytes.

L-leucine and L-arginine, at high doses, showed also a clear lipolytic effect in isolated idipocytes, indicating the possible importance of the dietary amino acid profile in the regulation of adiposity in this species.

IGF-I and IGF-II expression was not modified after incubation with GH, indicating that GH effects on lipolysis would be direct and independent of IGFs.

In summary, the present study contributes to improving knowledge on the regulatory role of GH and amino acids in rainbow trout adipocyte metabolism in order to optimize feed efficiency and diet composition for aquaculture.

Acknowledgements

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EXPOSURE TO DIFFERENT LIGHT SPECTRA AFFECTS THE ORGANIZATION OF THE BIOLOGICAL CLOCK DURING EARLY DEVELOPMENT AND METAMORPHOSIS OF SENEGALESE SOLE (*SOLEA SENEGALENSIS*)

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Our aim in this research was to determine the effects of light spectra on the profile expression of clock genes (*Per1*, *Per2*, *Per3* and *Clock*) during sole ontogeny (early development and metamorphosis). Rhythmic clock gene expression appears from the first 24 hours after fertilization. Cosinor analysis revealed a consistently rhythmic expression in sole embryos for *Per1* and *Per3* under LDB as well as for *Clock* under LDB and LDW. In contrast, under LDW condition *Per1*, *Per2* and *Per3* robust rhythms were not detected until larval stage 4dpf, when rhythms also reached their typical expression profiles. During metamorphosis, the phase of the rhythms was maintained. However, a significant decrease in mRNA expression levels was observed through the metamorphic stages. Rhythms under LDR condition were the most disturbed and *Per2* transcript levels were the most strongly affected by light spectra. In summary, our results suggest that LDB light-dark cycle accelerates molecular clock establishment and reinforces the importance of reproducing natural underwater photoenvironment (LD cycles of blue wavelengths) during incubation and early development of fish larvae for the optimal organization of a functional clock.

Introduction

Light information is a crucial environmental factor that controls the onset of functional clock machinery during fish development. Recent studies in the Senegalese sole reveal that photoperiod plays a key role in the early circadian system establishment and synchronization (1). In marine environments, the water column acts as a monochromatic filter that determines a light spectrum gradient with depth; thus, higher wavelengths from visible spectrum are gradually dissipated in superficial waters, whereas blue wavelengths can reach deeper waters which the sole inhabits. Previous studies demonstrated that light-dark cycles of blue wavelengths (LDB) during incubation of fertilized sole eggs improve the hatching rate. Moreover, LDB cycles during early development anticipate the beginning of eye pigmentation, mouth opening and pectoral fin development (2). In most cases, aquaculture practices are conducted under established protocols that differ considerably from natural environmental conditions. Therefore, research must be directed to improving artificial breeding and optimizing rearing conditions of the cultivated

species. In this sense, one of the main objectives should be to reproduce the natural conditions for each species. For this purpose, we have focused our work on determining light spectra effects in the biological clock organization during sole ontogeny.

Materials and Methods

1. Animals and experiment designs. Senegalese sole fertilized eggs were collected in darkness immediately after spawning during the night-time and acclimated to light-dark (LD) 12L:12D cycles of white (LDW), blue (LDB, λ peak=463nm) or red (LDR, λ peak=685nm) lights. Samples during early development (0, 1, 2, 4 days post-fertilization or dpf) and metamorphosis (9, 15, 25 dpf) were obtained every 4 hours (n=4; larvae pools=2-20 specimens).

2. Quantitative real-time PCR analysis. This was performed in a CFX Touch™ Real-Time PCR Detection System (Biorad). PCR reactions were developed in a 20 µl volume using cDNA generated from 1 µg of RNA. SensiFAST™ SYBR No-Rox kit (Bioline) and specific primers from sole *Per1*, *Per2*, *Per3* and *Clock* sequences were used. For normalization, sole *RPS4* was used as the housekeeping gene. The relative mRNA expression was determined by the ΔΔCt method.

Results and Discussion

1. Light-Dark cycles of white light (LDW). Under LDW condition, *Per1*, *Per2* and *Per3* rhythmic expression was detected from the first 24 hours post-fertilization. During the next 48 hours, the rhythmicity undergoes a gradual synchronization until larvae reach 4dpf, when *Per1* and *Per3* robust rhythms were clearly established, as reported in a previous study (1). *Per2* rhythmic oscillations were not consistently detected until early metamorphic stage (9dpf). In contrast, marked rhythmicity of the *Clock* gene appears from fertilized sole eggs. Typical acrophase values for all genes analyzed were reached at the end of early development (4dpf) and the phase was maintained during the metamorphic process.

2. Light-Dark cycles of blue wavelengths (LDB). Cosinor analysis revealed a robust rhythmic expression in sole embryos for *Per1*, *Per3* and *Clock* genes from the first day after fertilization (0dpf) under LDB condition (Fig. 1). *Per2* rhythmic oscillations were inconsistent during early developmental stages and consistent rhythms were reached at 9dpf. Nevertheless, our results show that the attaining of characteristic acrophases was advanced in LDB compared to LDW and LDR, reaching the typical expression profiles earlier under this condition. This result could be related to the early onset of the eye pigmentation under LDB condition during incubation of fertilized sole eggs. Moreover, a previous study in a zebrafish light-inducible cell line evidences that short wavelengths (blue and ultraviolet) produce optimal cryptochrome/blue-light photoreceptor stimulation after a monochromatic light exposure (3).

3. Light-Dark cycles of red wavelengths (LDR). Rhythms under LDR are the most disturbed. *Per 1*, *Per3* and *Clock* presented a similar rhythmic expression pattern to LDW. In contrast, *Per2* was strongly affected by LDR, not establishing a steady rhythm during sole ontogeny. The red light stimulus is not appropriate to

activate the photoreceptor pigments. This fact is in agreement with a previous study in which activity rhythms in LDB and LDW changed from diurnal to nocturnal on days 9dpf to 10dpf whilst sole larvae under LDR remained nocturnal during the ontogeny period (4).

Our findings underline the need to control the environmental factors in aquaculture practices to reproduce natural underwater photoenvironment.

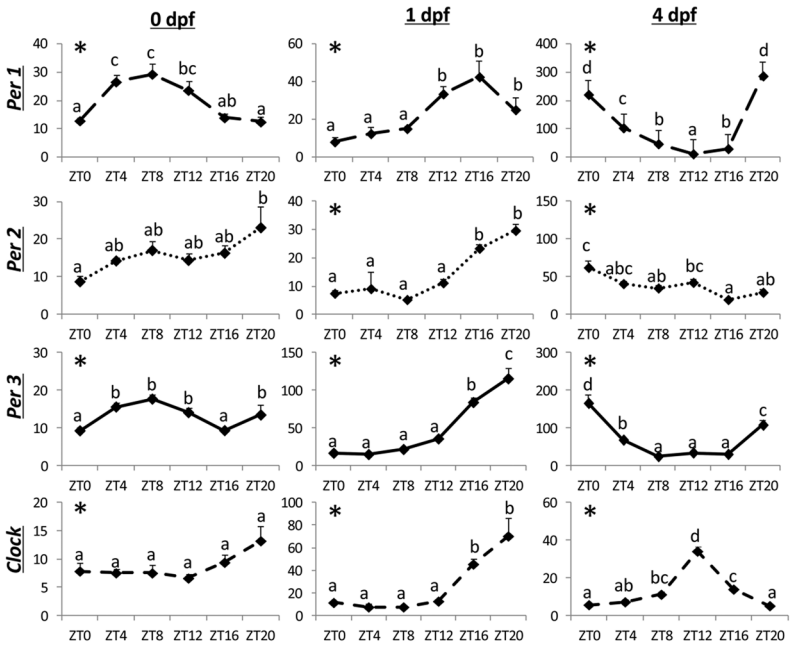

Figure 1. Relative mRNA expression of clock genes (--- *Per1*; ····· *Per2*; — *Per3*; ---- *Clock*) under light-dark cycles of blue wavelengths (LDB) during early development (0dpf, 1dpf, 4dpf). Different letters indicate significant statistical ANOVA differences (p≤0.05).Asterisks (*) represent daily rhythms significance analyzed by cosinor method.

Acknowledgements

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THE RETINA OF THE FLATFISH *SOLEA SENEGALENSIS* AS A CIRCADIAN CLOCK

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The retina of most vertebrate species is a remarkably rhythmic tissue that plays a key role in the circadian organization of animal physiology and behavior. Autonomous oscillators and photoreceptive capacities have recently been recognized in several retinal cell types different from photoreceptors, providing evidence that a network of circadian clocks is present within this neural tissue. In this study, we have performed *in situ* hybridization to localize *Per1*-, *Per2*-, *Per3*-, *Clock*- and *Hiomt*-expressing cells in the Senegalese sole retinas with the aim of identifying putative clock cells in this species. Our results revealed that the four above clock genes together with *Hiomt* were mainly expressed in the photoreceptor cells of the outer nuclear layer (ONL). Moreover, *Per1*, *Per3* and *Clock* transcripts were localized in the inner nuclear layer (INL), whereas a specific labeling for *Per3* and *Hiomt* was found in neurons from the ganglion cell layer (GCL). These data point to photoreceptors as strong candidates to contain an autonomous circadian clock in the retina of sole.

Introduction

The retina represented the first robust demonstration of an autonomous circadian clock outside the suprachiasmatic nucleus (SCN) of the hypothalamus (1). A multitude of rhythms are generated within the eye, allowing the organism to anticipate and adapt to the 24 h change in light intensity, thereby optimizing visual function for each photic situation (2). Due to its high heterogeneity and multi-layered nature, with many different types of specialized cells, the precise localization of the clock(s) that control these rhythms remains unclear, especially in fish. In this context, the Senegalese sole emerges as an interesting model for retinal studies since several genes implicated in the circadian axis show robust daily oscillations in this organ, suggesting the existence of a potential primary pacemaker (3). Here we go deeper into the knowledge of the circadian clock of the sole retina by localizing, for the first time in a marine fish species, clock gene-expressing cells within the different retinal cell layers.

Materials and Methods

Adult Senegalese sole specimens were maintained under natural photoperiod conditions (36° 31' North), constant temperature and salinity (19 ± 1 ºC and 39 ppt, respectively). In April and during day time, they were anesthetized and intracardiacally perfused with 0.85% saline solution followed by Bouin's fixative. Eyes

were excised, fixed overnight in the same fixative at 4 ºC and embedded in paraffin. Transverse sections of 7 µm-thick were obtained with a microtome and mounted on Polysine™ slides for *in situ* hybridization (ISH) processing. For clock genes, antisense and sense radioactive riboprobes were generated and for *Hiomt* expression, digoxigenin-labeled riboprobes were used. Double opsin and tyrosine hydroxylase (TH) immunostaining was carried out on sections previously processed by ISH. All procedures were performed as described elsewhere (4), with minor modifications.

Results and Discussion

1. Clock genes and *Hiomt*-expressing cells in sole retina. *Per1* radiolabeling was particularly concentrated in the outer nuclear layer (ONL) containing the cell bodies of photoreceptor cells (Fig. 1A). Specific hybridization with the antisense probe was also observed in scattered cells located in the inner nuclear layer (INL), which contains amacrine, horizontal and bipolar neurons, although showing a lower density of silver grains than in ONL (Fig. 1B). *Per1* labeling was not detected in neurons of the ganglion cell layer (GCL). In a similar manner, *Per2*-expressing cells were detected primarily in the ONL, but not in the INL or GCL (Fig. 1C). *Per3*-expressing cells were found in the three cellular layers of the retina (Fig. 1E-H) and *Clock* transcripts were revealed throughout the ONL, although groups of cells showing a specific hybridization signal were also observed in the INL (Fig. 1I, J). No conspicuous *Clock* radiolabeling was evident in cells of the GCL. Regarding *Hiomt* expression, a specific labelling was revealed in photoreceptor cells (ONL) and in the ganglion cells (GCL) (Fig. 1, K, L). No signal was detected in control sections incubated with the sense riboprobes (Fig. 1, D and data not shown).

2. Opsin and TH immunoreactivity in sole retina. An intense opsin-immunostaining was found in the outer segments of photoreceptor cells. Some scattered opsin-immunoreactive cells were also identified in the INL. Double immunostaining showed that these cells received varicose nerve terminals from TH-immunopositive fibers running along the INL. In turn, TH cells were identified in the INL of sole retina, being small in size and exhibiting rounded cell bodies (data not shown).

According to *Xenopus* and avian models, our results indicate that photoreceptors of the retinal ONL contain all the components of a complete circadian system, as these cells concentrate opsin immunofluorescence (photoentrainment pathway), *Per1*-, *Per2*-, *Per3*-, *Clock*-expressing cells (circadian clock) and *Hiomt*-expressing cells (melatonin, output pathway) (5, 6). Further studies, such as laser capture microdisection would be needed to confirm their role as autonomous circadian oscillators. However, groups of cells in the INL and GCL showed specific hybridization for some clock genes and/or *Hiomt* that could correspond, at least in part, to photoreceptor and/or dopaminergic cells, as shown by the opsin and tyrosine hydroxylase immunostaining found in this layer. Thus, the photoperiodic control of melatonin production might not be exclusively linked to photoreceptors, and the existence of a multioscillatory network involving different cell layers should not be ruled out.

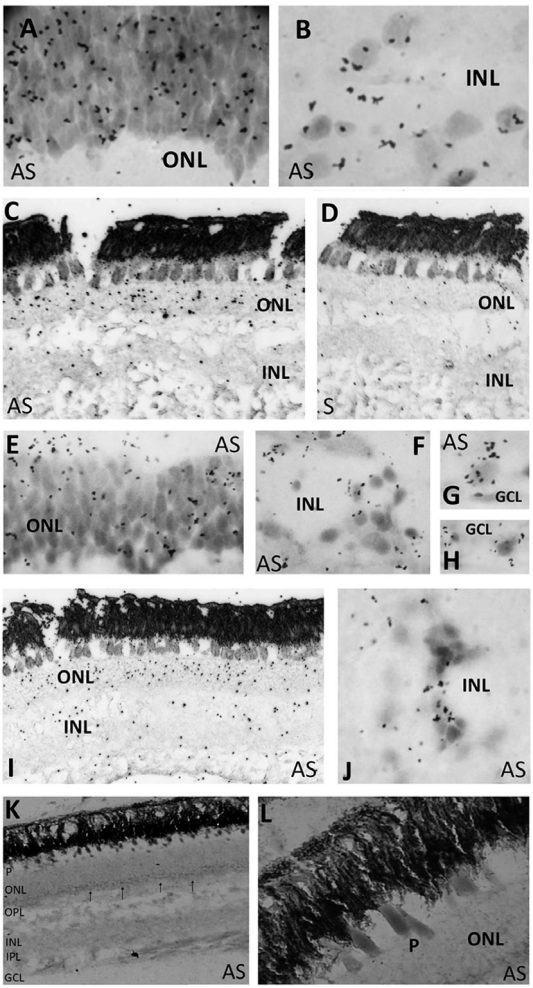

Figure 1. Retinal localization of clock genes and *Hiomt* transcripts in Senegalese sole revealed by ISH. Bright-field retinal sections hybridized with *Per1* (A, B), *Per2* (C), *Per3* (E-H), *Clock* (I, J) and *Hiomt* (K, L) antisense riboprobes. Specific radiolabeling for the four clock genes together with *Hiomt* was found in photoreceptor cells (A, C, E, I, K, L). *Per1*-, *Per3*- and *Clock*- expressing cells were also identified in inner neurons (B, F, J). Control section hybridized with *Per2* sense riboprobe showing no specific signal (D). Abbreviations: AS, antisense riboprobe; S, sense riboprobe; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer Pc, photoreceptor cells.

Acknowledgements

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TESTICULAR ORGAN CULTURE FOR THE STUDY OF SEA BASS SPERMATOGENESIS

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The role of retinoic acid (RA) in the induction of meiosis has been demonstrated in humans, rodents, birds, and amphibians, but studies in fish are still very scarce. The present work was aimed at the study of the gene expression that some key players of this pathway may have in the onset of meiosis in the European sea bass (*Dicentrarchus labrax*). Testicular explants were cultured in the presence of DEAB, an RA synthesis inhibitor or an excess of all-trans RA (ATRA). The study showed differences in the expression of several genes involved in various steps of the RA signaling pathway.

Introduction

The initiation of meiosis can be considered to be the signal that triggers the onset of spermatogenesis. The key function of retinoic acid (RA) in the regulation of spermatogenesis has been shown in higher vertebrates, but its role in fish is still a matter of debate. RA is synthesized from retinol by two reactions involving alcohol dehydrogenases (adh) and aldehyde dehydrogenases (aldh). Two proteins participate in its transport: retinol binding protein (rbp) and RA binding protein (rabp), whereas the final RA concentration in the cell is regulated by cyp26, a member of the P450 family enzymes (1). RA acts as a ligand for its receptors, RAR and RXR, which in turn binds to RA Response Elements (RARE), regulating the transcription of several genes (2) (Fig. 1). Among these genes, it is worth mentioning *stra8*, responsible for the onset of spermatogenesis in humans, rodents, birds and amphibians. In fish, the information about this pathway is still scarce (3) and only recently was *stra8* identified in the Southern catfish (4). The purpose of this study is to set up an explant culture method for the assessment of RA-related gene expression and contribute to the basic knowledge of the role of RA in the onset of fish spermatogenesis.

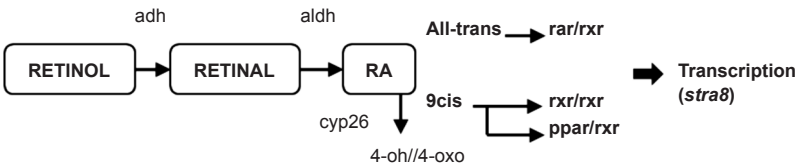

Figure 1. Schematic representation of the RA signaling pathway.

Materials and Methods

Testes from one-year-old sea bass (412 and 420 days post hatching) were separated left from right, and immediately immersed in SBR buffer, washed and finally

placed in a culture system consisting of a nitrocellulose filter over an agarose cylinder previously embedded in supplemented L-15 medium modified from (5). The explants were treated with DEAB (1 µM), or with ATRA (100 nM) and incubated at 21°C for 48 h in a dark humid chamber. Both compounds were dissolved in DMSO and the possible effects of the solvent were also checked. Gonadal explants were of a small size and special care was taken to maintain the tissue always in contact with the surface of the nitrocellulose filter and to facilitate a proper oxygen exchange throughout the tissue. At the end of the incubations, the testes were collected for histology and mRNA extraction. The expression of several genes involved in the RA signaling pathway was assessed by real time qPCR.

Results and Discussion

Histological examination of the gonads showed no apparent differences in development between each treatment and its respective control. Moreover, testes were all at the same developmental stage, corresponding to Stage I, and characterized by the presence of spermatogonia (6).

The RA signaling pathway found in invertebrates and vertebrates interacts with different biological processes including cell proliferation, differentiation and apoptosis by regulating the expression of several genes (7). The present study shows that both experimental treatments related to the RA signaling pathway modified the expression of *pcna*, a marker of cell proliferation, with DEAB increasing *pcna* gene expression whereas ATRA resulted in a decrease of *pcna* expression, suggesting a possible effect on the onset of meiosis (Fig. 2).

The *cyp26* gene belongs to the p450 family and metabolizes RA into its non-active hydroxylated polar derivatives (8). The excess of RA, induced by the addition of ATRA, resulted in an increase of testicular *cyp26* expression. This increase is consistent with its function as RA metabolizing enzyme and inactivates the excess of RA present in the gonad that could be toxic for the tissue (Fig. 2).

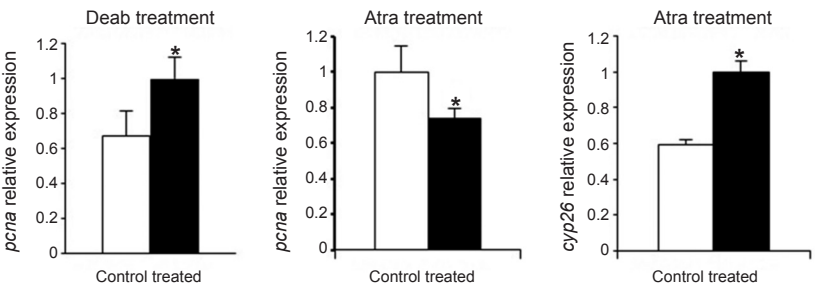

Figure 2. *pcna* and *cyp26* relative gene expression in gonadal explants incubated in the presence of DEAB or ATRA. Asterisks denote significant differences relative to the control group ($p < 0.05$; T-test).

In our experimental culture system, there is no added source of extragonadal retinol or retinal, therefore the gonad alone is responsible for dealing with the excess (ATRA treatment) or deficiency (DEAB treatment) of RA in order to regulate

the homeostasis of the pathway. Both treatments had an effect on the expression of several genes involved in the RA signaling pathway; the inhibition treatment resulted in an increasing trend of expression in the majority of the studied genes, whereas the opposite pattern was found in presence of an excess of RA (Table 1).

The study also concluded that the explant culture method is suitable for the assessment of the RA signaling pathway for a short term period when the gonad tissue is too small. Moreover, DMSO can be used as a suitable carrier for the tested compounds since it had no effect on the expression levels of any of the studied genes.

Table 1. Expression patterns of several genes involved on the RA signalling pathway.

Expression	DEAB	ATRA
Upregulated	aldh1a2, rarα, rxrα, pparγ, stra6	
Downregulated		aldh1a2,aldh1a3, rarα, rxrα, pparγ, stra6
No expression	rabp	rabp
No differences	aldh1a3, rbp4	rbp4

Gene nomenclatures: *aldh1a2*- aldehyde dehydrogenase 1a2, *aldh1a3*- aldehyde dehydrogenase 1a3, *rarα*- RA receptor alpha, *rxrα*- retinol receptor alpha, *pparγ*- peroxisome proliferator-activated receptor gamma, *stra6*- stimulated by retinoic acid 6, *rabp*- retinoid acid binding protein, *rbp4*- retinol binding protein 4.

Acknowledgements

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CORTISOL TREATMENT INDUCES THE EXPRESSION OF *gPer1a* IN *CARASSIUS AURATUS*: *IN VITRO* AND *IN VIVO* EVIDENCE

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The daily rhythm of plasma cortisol levels is considered to be an output of the circadian system. Such a rhythm could synchronize peripheral clocks acting also as an input of the circadian system. However, the possible role of glucocorticoids as a signal that leads to a temporal organization of the different endogenous clocks has not been studied in fish. To go deeper into this role of glucocorticoids as a temporal messenger, we investigate the effect of cortisol treatment on the expression of clock genes in goldfish (*Carassius auratus*) by using *in vitro* and *in vivo* approaches. 1) *In vivo*: goldfish (under 12L:12D photoperiod and fed at ZT 2) were intraperitoneally injected with cortisol (0.1 and 1 µg/g bw), and hypothalamus, liver and muscle were sampled at 2 h post-injection. 2) *In vitro:* livers were short time (4-5 h) cultured with cortisol (0, 0.1, 1 and 10 µM) or dexamethasone (0.1, 1, 10 and 100 nM). The expression of some clock genes was quantified by RT-qPCR in both experiments. Cortisol increased the expression of *gPer1a* in liver cultures while dexamethasone not only induced *gPer1a*, but also decreased *gBmal1a* expression. *In vivo,* both doses of cortisol induced *gPer1a* expression in the hypothalamus, but only one dose induced *gPer1a* expression in muscle (0.1 µg/g bw) and the liver (1 µg/g bw). These results show that the treatment with glucocorticoids can modify clock gene expression in goldfish, supporting the idea that cortisol could play a role in the functional organization of the circadian system in fish.

Introduction

At present, it is assumed that the circadian system of teleosts is composed by a network of oscillators located in the whole body. However, the mechanisms underlying the synchronization of central and peripheral clocks have not been deeply studied. Many hormones have been proposed to be the signals that interconnect the network of oscillators, glucocorticoids being one of the most studied candidates. The existence of a rhythm of glucocorticoids in some vertebrates from teleost (1) to mammals (2) has been suggested. Moreover, previous studies focused on the possible role of glucocorticoids as signals that modulate clock gene rhythms in mammals showed that a treatment with these hormones in cultured cells or living animals increased the expression of the clock gene *gPer1* (2). In goldfish, clock genes have been cloned and their rhythmic expression profiles have been described in some central and peripheral locations including the liver (3). In the present study, we investigate the role of glucocorticoids as modulators of clock genes in the hypothalamus and liver of goldfish.

Materials and Methods

Fish were maintained under a 12L:12D photoperiod and fed 2 h after the onset of light (*Zeitgeber Time*, ZT2). For the *in vivo* experiment, goldfish (8.5 ± 0.5 g body weight, bw) were divided into four experimental groups and intraperitoneally injected with cortisol (1 µg/g bw or 10 µg/g bw) or saline (plus vehicle 9.2 % ethanol for the higher cortisol doses). Fish were injected at ZT8 and sacrificed 2 h post-injection. Hypothalamus, muscle and liver were sampled and *gPer1a* expression was quantified by qRT-PCR as previously described (3). Specific primers are shown in Table 1. For the *in vitro* approach, fish maintained as described above and fasted for 24 h, were sacrificed at ZT2. Livers were preincubated in 2 ml of *Dulbecco's Modified Eagle Medium* (DMEM, 15 mg liver/well) for 2 h. Then, the medium was replaced by medium alone (control group), with different concentrations of cortisol (0.1, 1 and 10 µM) or dexamethasone (0.1, 1, 10 and 100 nM). Liver samples were collected after 4 h or 5 h (for cortisol and dexamethasone treatments, respectively) and *gPer1a* and *gBmal1a* expression was measured by qRT-PCR. The relative mRNA expression was determined by ΔΔCt method (3).

Table 1. Accession numbers of the genes and primers sequences used in quantitative qRT-PCR studies.

Gen	Accession number		Sequence (5'-3')	Product size (bp)
gPer1a	EF690698	Forward	CAGTGGCTCGAATGAGCACCA	155
		Reverse	TGAAGACCTGCTGTCCGTTGG	
gBmal1a	(4)	Forward	ATCGATGAGTCGTTCCCGTG	161
		Reverse	AGATTCTGTTCGTCTCGGAG	

Results and Discussion

In vivo, the injection of the low cortisol dose (1 µg/g bw) significantly induced *gPer1a* in the hypothalamus and liver of goldfish, while in the muscle, the mRNA increment was not statistically significant. The highest dose of cortisol tested (10 µg/g bw) induced *gPer1a* in the hypothalamus and muscle, but had no effect on the liver (Fig. 1).

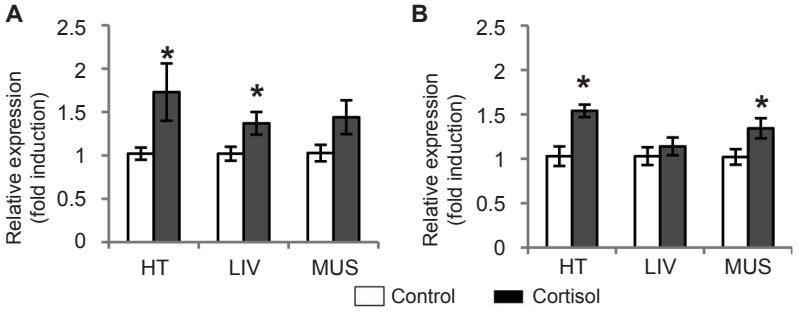

Figure 1. *gPer1a* expression in hypothalamus (HT), liver (LIV) and muscle (MUS) of goldfish at 2 h post-injection. A) Cortisol 1 µg/g bw. B) Cortisol 10 µg/g bw. Results are expressed as the mean ± SEM (n=7). * $p<0.05$ (t-student).

Similarly, the cortisol treatment during 4 h increased the expression of *gPer1a* gene in liver cultures in a concentration dependent-manner, being significant at 1 μM (Fig. 2A). Cortisol in the culture medium did not modify the expression of *gBmal1a* (Fig. 2B). The exposure to dexamethasone for 5 h also induced *gPer1a* in a concentration dependent manner and furthermore decreased significantly the expression of *gBmal1a* in the hepatic cultures (Figs. 2C and D).

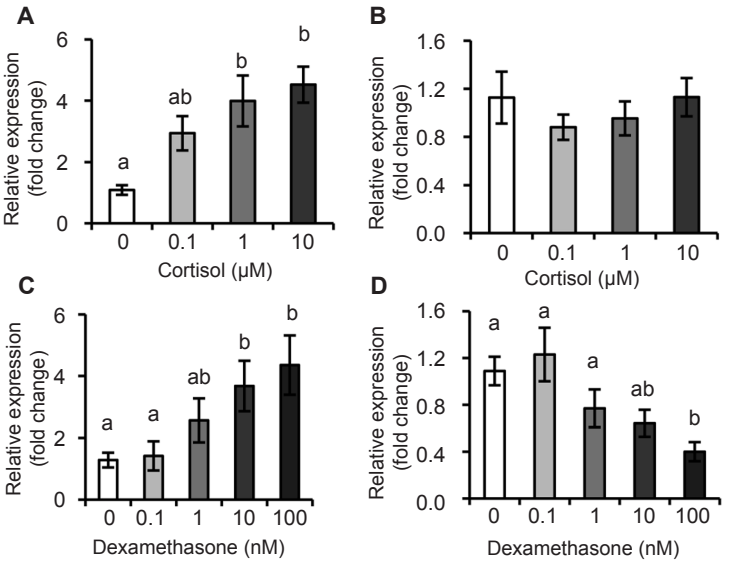

Figure 2. Clock gene expression in goldfish hepatic cultures. *gPer1a* (A) and *gBmal1a* (B) expression in liver exposed to cortisol. *gPer1a* (C) and *gBmal1a* (D) expression in liver exposed to dexamethasone. Results are expressed as the mean ± SEM (n=6). Different letters indicate significant differences among groups (SNK test).

Present results demonstrate that glucocorticoids modulate clock gene expression in teleosts. This agrees with data obtained in mammals in which a glucocorticoid element response has been found in the promoter of *Per1* gene that could be well conserved (2).

Overall, present results suggest that glucocorticoids could be involved in the functional organization of the circadian system in goldfish. Cortisol could be acting as an endogenous signal that would synchronize not only peripheral oscillators (as suggested in mammals), but also central ones (present findings in hypothalamus).

Acknowledgements

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LIGHT AND FEEDING INFLUENCE ON DAILY VARIATIONS OF CLOCK GENES AND LIVER METABOLISM IN RAINBOW TROUT

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Most physiological processes, including feeding behaviour and energy metabolism, exhibit circadian (i.e. 24 h) rhythmicity, which may play a major role in maintaining functional homeostasis. Metabolic homeostasis relies on accurate and collaborative circadian timing within individual cells and tissues all over the body. These rhythms are regulated by the circadian clock system, which involves a set of "clock genes". While the *master clock* is localized in central nervous system-related structures, different peripheral locations, such as the liver, have been recently described for clock gene expression in most vertebrates, including fish. A reciprocal influence between both energy metabolism and circadian system has been described in mammals, but little is known in this respect regarding other vertebrates. In order to characterize such interaction in teleosts we subjected rainbow trout to different lighting and nutritional conditions. The daily profile of expression for several energy metabolism-related genes and clock genes were assessed in the liver of trout sacrificed at different time points in 24 h daily cycles. Most of the genes assessed, including those related to glucose (*PEPCK*, *G6Pase*, *GPase*, *GK*, *PK*, and *GLUT2*) and lipids metabolism (*FAS*), were not significantly affected by environmental cues such as light or food, but a cue-dependent phase shift was observed for G6Pase, PK and FAS expression. In addition, the daily rhythm of clock gene (*clock1a*, *bmal1* and *per1*) expression was mostly influenced by light in such a way that exposing animals to constant darkness did completely disrupt their typical profile. Such variation in the clock gene expression may be in part responsible for changes observed in the expression of genes related to energy metabolism. Taken together our data support the involvement of the circadian system in regulating the daily expression profile of energy metabolism-related genes in rainbow trout liver.

Introduction

Biological rhythms play an important adaptive role in the temporal organization of living organisms, allowing them to adjust their physiological processes to the chronological arrangement of the external world (1, 2). Fish are no exception and a variety of studies demonstrate the existence of both daily and yearly rhythmic fluctuations in biochemistry, metabolism and behaviour in several fish species (3). In particular, metabolic homeostasis relies on accurate and collaborative circadian timing within individual cells and tissues all over the body. These rhythms are regulated by the circadian clock system, which involves a set of "clock genes". While the *master clock* is localized in central nervous system-related structures, different peripheral locations, such as the liver, have been recently described for clock gene expression in most vertebrates, including fish. A reciprocal influence

between both energy metabolism and circadian system has been described in mammals, but little is known about this in other vertebrates. In order to characterize such interaction in teleosts we subjected rainbow trout to different lighting and nutritional conditions. Thus, the present study describes the influence of light and food on the daily profile of liver metabolism in rainbow trout by assessing the expression profile of clock genes and energy-related metabolism genes in such tissue.

Materials and Methods

After the acclimatization to a 12L:12D photoperiod and feeding time at ZT2 (ZT0: Lights on) three cohorts of rainbow trout (N=56 each) were subjected to different lighting and nutritional conditions (LD+Food, DD+Food, LD+Fasting) and then sacrificed every 4 h during a 24 h light/dark cycle (n=8/time point), starting at ZT0. Thus, scheduled sampling time points were ZT0, ZT4, ZT8, ZT12, ZT16, ZT20 and ZT0´ of the following day. Liver was collected in order to assess the daily profile of expression for clock genes (*clock1a*, *bmal1*, *per1*) and several energy metabolism-related genes (*PEPCK*, *G6Pase*, *GPase*, *GK*, *PK*, and *GLUT2*).

Results and Discussion

The daily rhythm of clock gene expression in rainbow trout liver (Fig. 1) appeared to be mostly influenced by light in such a way that exposing the trout to constant darkness did completely disrupt their typical profile by phase shifting the time of the daily peak. The same tendency was observed for the expression profile of those enzymes involved in melatonin (*aanat1*, *aanat2*, *and hiomt*), with the latest being also influenced by food (Table 1).

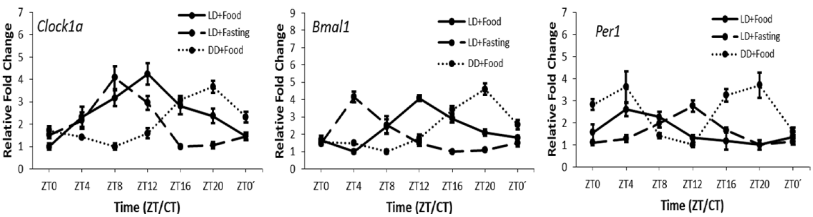

Figure 1. Daily profile of a) *Clock1a*, *Bmal1*, and *Per1* mRNA expression in liver of rainbow trout subjected to different lighting and nutritional conditions (LD+Food, DD+Food, or LD+Fasting). Expression results were normalized by β-actin mRNA levels. Each value represents the mean ± S.E.M. of four fish.

Most of the assessed genes related to glucose (*PEPCK*, *G6Pase*, *GPase*, *GK*, *PK*, and *GLUT2*) and lipids metabolism (*FAS*) in the liver were not significantly affected by any of the environmental cues assayed (light and food), but a cue-dependent phase shift was observed for *G6Pase*, *PK* and *FAS* expression as shown in the acrophase data (Table 1).

The herein reported variation of the expression profile of clock genes might be in part responsible for the changes observed in the expression profile of metabolism-related genes in rainbow trout liver. Taken together our data support the in-

volvement of the circadian system in the regulation of the daily expression profile of energy metabolism-related genes in rainbow trout liver.

Table 1. Parameters defining the periodic sinusoidal functions determined by the cosinor analysis for metabolism-related (*PEPCK*, *PK*, *GK*, *FAS*, *G6Pase* and *GLUT2*) and melatonin synthesis-related genes (*AANAT1*, *AANAT2* and *HIOMT*) in liver of rainbow trout subjected to different lighting and nutritional conditions. Expression results were normalized by β-actin mRNA levels. Each value represents the mean ± S.E.M. of four fish. * Noise/Signal (N/S) < 0.3. The profile fits to a 24 h rhythm in the cosinor analysis.

Gen	Date	LD + Food	DD + Food	LD + Fasting
PEPCK	Mesor	2.56	4.35	1.95
	Amplitude	1.83	3.99	1.11
	Acrophase (ZT/CT)	1.50	0.25	5.25
	N/S	0.23*	0.22*	0.17*
PK	Mesor	4.14	2.23	2.15
	Amplitude	2.98	1.06	1.07
	Acrophase (ZT/CT)	11.25	12.50	6.50
	N/S	0.21*	0.24*	0.17*
GK	Mesor	27.70	39.27	5.14
	Amplitude	27.12	38.54	4.62
	Acrophase (ZT/CT)	3.25	4.00	5.50
	N/S	0.29*	0.20*	0.17*
FAS	Mesor	6.90	2.87	6.99
	Amplitude	5.11	1.70	4.42
	Acrophase (ZT/CT)	23.25	23.25	10.50
	N/S	0.25*	0.19*	0.18*
G6Pase	Mesor	1.87	1.79	1.68
	Amplitude	0.65	0.77	0.60
	Acrophase (ZT/CT)	8.50	15.75	16.50
	N/S	0.23*	0.16*	0.16*
GLUT2	Mesor	2.17	1.76	1.72
	Amplitude	1.34	0.62	0.65
	Acrophase (ZT/CT)	6.25	5.50	8.50
	N/S	0.15*	0.20*	0.17*
AANAT1	Mesor	1.88	2.03	1.88
	Amplitude	0.62	1.09	0.75
	Acrophase (ZT/CT)	11.10	19.30	11.44
	N/S	0.21*	0.15*	0.17*
AANAT2	Mesor	2.79	2.45	3.12
	Amplitude	1.56	1.47	2.32
	Acrophase (ZT/CT)	12.48	20.08	11.45
	N/S	0.19*	0.16*	0.17*
HIOMT	Mesor	4.69	3.07	4.70
	Amplitude	4.34	2.07	4.27
	Acrophase (ZT/CT)	10.04	18.42	16.50
	N/S	0.18*	0.16*	0.17*

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METABOLIC EFFECTS OF THE THYROID AND INTERRENAL SYSTEMS ON THE LIVER OF GILTHEAD SEA BREAM (*SPARUS AURATA*)

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The final products of the hypothalamus-pituitary-thyroid and hypothalamus-pituitary-interrenal axes affect energy metabolism in teleost fish. The purpose of this study was to determine the effects of both hormonal systems on hepatic intermediary metabolism in gilthead sea bream (*Sparus aurata*) juveniles. The following compounds were administered for 35 days: i) 3,5,3'-triiodothyronine (T3), the biologically active thyroid hormone; ii) propylthiouracil (PTU), an antithyroid drug which inhibits the synthesis of thyroid hormones; iii) cortisol, a steroid hormone that is released in response to stress; and iv) dexamethasone, a synthetic glucocorticoid that binds to cortisol receptors and evokes similar effects, but of higher intensity. Growth parameters and changes in hepatic metabolic reserves were analyzed in the specimens. In addition, several hepatic enzymatic activities related to the metabolism of carbohydrates, lipids and proteins [pyruvate kinase (PK), glycerol-3-phosphate dehydrogenase (G3PDH) and aspartate transaminase (GOT)] were also assessed. Our results show an increase in glycolysis in the dexamethasone treated group, but not in the others. Lipid mobilization was stimulated in all experimental groups, whereas protein metabolism was affected in part by the effect of T3, PTU and cortisol. These results indicate an unclear differential response between T3 and PTU groups, suggesting a possible metabolic compensation to address the decline in the production of T3 due to the administration of PTU. Moreover, dexamethasone enhances cortisol effects, increasing catabolic metabolic reserves.

Introduction

In fish, the hypothalamus-pituitary-interrenal (HPI) and the hypothalamus-pituitary-thyroid (HPT) axes are involved in a wide range of metabolic processes, and usually have been studied independently to define the effects they cause on fish physiology (1). Cortisol, the most important hormone produced by the HPI axis, and thyroid hormones (T3 and T4, THs) from the HPT, affect diverse high energy cost physiological processes such as growth or osmoregulation (2). Nevertheless, other studies pointed to an interaction between both endocrine axes, which apparently have common mechanisms of regulation (3). In our study, we assessed the hepatic metabolic effects that final products of HPI and HPT axes (cortisol and THs respectively) produce in gilthead sea bream (*Sparus aurata*). T3 stimulates glycogenolysis and gluconeogenesis in some species (4); cortisol also presents a hyperglycemic effect (1). In *S. aurata*, cortisol also influences the metabolism of carbohydrates, proteins and lipids, as well as the activity of some enzymes involved in these processes, such as pyruvate kinase (PK) or glycerol-3-phosphate dehydrogenase (G3PDH) (5).

The objective of this study is to compare the metabolic effects caused by these endocrine products (T3 and cortisol) on hepatic intermediary metabolism of *S. aurata* through the oral administration of these hormones and their antagonists (PTU and DXM).

Materials and Methods

Juveniles of gilthead sea bream (*S. aurata*) (N=120), with an initial weight of 18.1 ± 0.2 g, were distributed in 5 tanks of 400 L (n=24 specimens per group). Tanks were maintained in a flow-through system with a salinity of 40 ppt and a temperature of 19 ºC. For 35 days, the compounds were administered orally to the animals, with the following doses: i) T3, 10 mg kg feed^{-1} (6); ii) PTU, 5 mg kg fish^{-1} day^{-1} (Ruiz-Jarabo, data not published); iii) cortisol, 400 mg kg feed^{-1} (7) and iv) DXM, 300 mg kg feed^{-1} (8). At the end of the exposure period, growth parameters (weight) were analyzed and liver samples collected to measure the enzymatic activity of pyruvate kinase (PK), glycerol-3-phosphate dehydrogenase (G3PDH) and aspartate transaminase (GOT) as in Polakof et al. (2006) described (9).

Results and Discussion

Growth of juvenile specimens was affected only in the DXM group (Table 1). This could be related to an enhancement of carbohydrate and lipid catabolism, as shown by the PK and G3PDH activity increases (Fig. 1). In addition, lipid mobilization was stimulated in all experimental groups, but this activation was also higher in the DXM group, as seen by G3PDH activity (Fig. 1). Protein metabolism was affected in part by T3, PTU and cortisol, as seen by a lower GOT activity (Fig. 1).

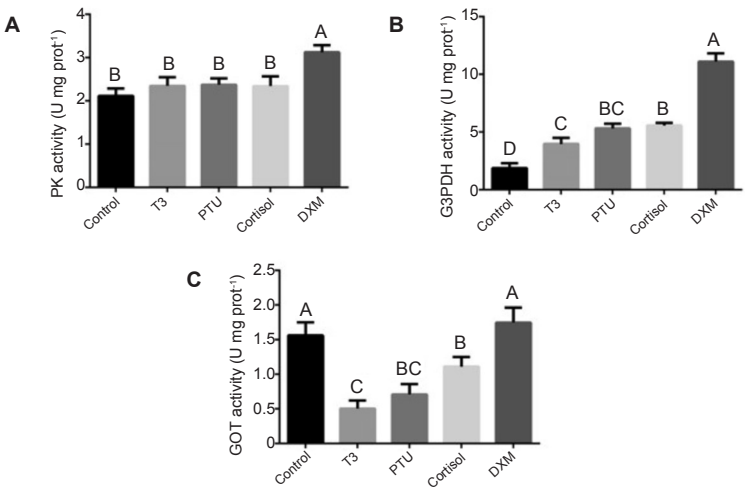

Figure 1. Liver enzymatic activities (U mg prot^{-1}) after 35 days of treatment. (*A*) Pyruvate kinase, PK activity; (*B*) Glycerol-3-phosphate dehydrogenase, G3PDH activity and (*C*) Aspartate transaminase, GOT activity. Letters indicate statistical different groups (one – way ANOVA, p<0.05).

A clear differential response between T3 and PTU groups could not be seen, suggesting a possible metabolic compensation to address the decline in T3 production due to PTU administration.

Finally, our results suggested the need for further studies involving the use of molecular biology tools in order to define how the changes in energy metabolism are induced by both thyroid and stress systems in *Sparus aurata.*

Table 1. Weight (g) of the gilthead sea bream (*S. aurata*) juveniles after 14 and 35 days of treatment (mean ± SEM). * indicates statistical differences between groups at day 14, and ** statistical differences between groups at day 35 (one-way ANOVA for each sampling point, $p<0.05$).

Days	Control	T3	PTU	Cortisol	DXM
0	18.8 ± 0.5	17.6 ± 0.4	17.4 ± 0.4	18.7 ± 0.6	17.8 ± 0.5
14	27.1 ± 0.8	25.1 ± 0.7	24.2 ± 0.7	25.0 ± 0.9	21.1 ± 0.7*
35	35.5 ± 1.1	35.5 ± 1.1	32.8 ± 0.9	32.6 ± 1.3	23.6 ± 0.8**

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ACTION OF KISSPEPTIN ON IN VITRO GONADOTROPIN RELEASE IN THE EUROPEAN SEA BASS (*DICENTRARCHUS LABRAX*)

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Kisspeptins are key players in the neuroendocrine control of puberty and other reproductive processes in mammals. It has been suggested that the effect of kisspeptins occurs exclusively at the level of hypothalamic GnRH secretion, since the stimulatory effect of kisspeptin on gonadotropin release is blocked by a GnRH antagonist. However, several studies have demonstrated that the KiSS/GPR54 system is expressed by the gonadotrophs, but *in vitro* studies assessing the direct stimulatory effects of kisspeptin on gonadotropin secretion in the pituitary have given conflicting results. In this study, we aimed to investigate the direct effect of sea bass kiss1 and kiss2 on the pituitary. The highly active peptides kiss1-15 and kiss2-12 were used to stimulate dispersed sea bass pituitary cells obtained from mature males. The peptide kiss2-12 induced LH release after 8 h of incubation, while the same concentration of this peptide evoked FSH release 4 h post-stimulation. However, kiss1-15 had no effect on LH or FSH release. On the other hand, kiss2-12 had a synergic effect with GnRH on LH release and, in the presence of sexual steroids (estradiol and testosterone), the stimulatory effect of kiss2-12 on LH and FSH release was reduced.

Introduction

The importance of GPR54 and its cognate ligands, kisspeptins, products of the *KiSS-1* gene, in the control of reproductive function in mammals has been substantiated by numerous experimental studies. The hypothalamus represents the primary site of action of kisspeptins on reproduction by regulating GnRH secretion; but a direct effect of kisspeptins at the pituitary level has also been suggested. However, *in vitro*, kisspeptin treatments of mammalian pituitary cells resulted in contradictory effects, with no stimulation or stimulation of gonadotropin release. It is therefore suggested that kisspeptins might act as endocrine/autocrine/paracrine signals in modulating hormonal secretion of the pituitary. In this study, our objective was to investigate the direct effect of sea bass kiss1 and kiss2 on the pituitary.

Materials and Methods

Pooled pituitaries from male European sea bass were excised in 1 mm^3 fragments. Cells were enzymatically dispersed using a trypsin/DNaseII digestion method modified from the method of Chang et al. (1). After estimating the number of viable cells by Trypan blue, cells were cultured in 24-well culture plates at a density of 3 x 10^5 cells/well/ml for 3 days at 20 ºC. At day 3, medium was replaced with serum-free culture medium (0.5 ml/well) containing or not containing freshly diluted hormones: A) kiss1-15 or kiss2-12 (10^{-6} M) for 2, 4, 6, 8 and 12 h. B) kiss1-15 or kiss2-12 (from 10^{-8} M to 10^{-5} M) for 4 and 8 h. C) kiss1-15 or kiss2-12 alone

(10^{-6} M) or in combination with GnRH (10^{-10} M) for 4 and 8 h. D) kiss1-15 or kiss2-12 (10^{-6} M) with or without estradiol (50 nM) or testosterone (50 nM) for 4 and 8 hours. One day before, the experiment cells were preincubated overnight with medium containing estradiol (50 nM) or testosterone (50 nM).

Results and Discussion

The time course studies of kiss1-15 and kiss2-12 effects on gonadotropin release are shown in Figure 1. Thus, in the presence of kiss2-12, a peak of LH release was observed after 8 h incubation, while the same concentration of this peptide induced a significant release of FSH in 4 h. The stimulatory effect of kiss2-12 on LH release is clearly more potent than the effect on FSH release. The peptide kiss1-15 had no effect on LH or FSH release.

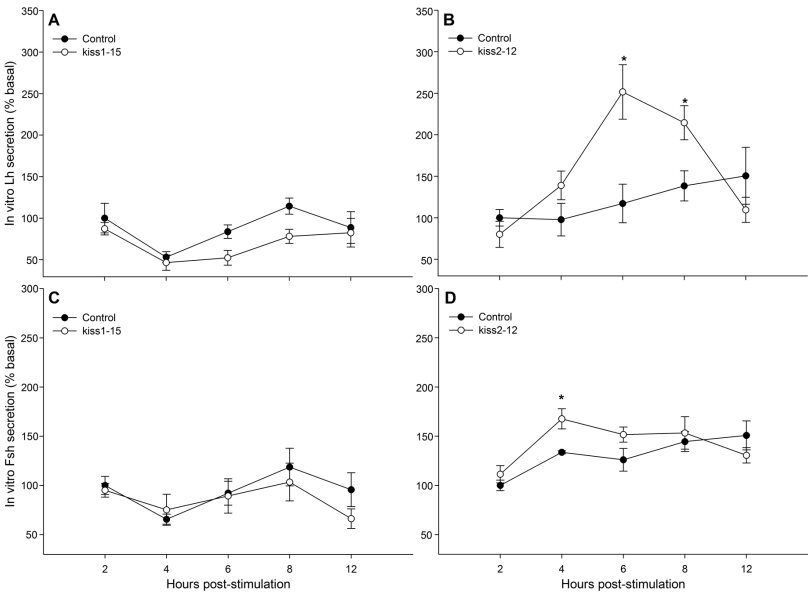

Figure 1. Time course of *in vitro* LH release after kiss1-15 (A) or kiss2-12 (B) treatments at 10^{-6} M. Time course of *in vitro* FSH release in response to kiss1-15 (C) or kiss2-12 (D) at 10^{-6} M. Results are expressed as a percentage increase over basal secretion. Significant differences between groups are represented by an asterisk above each sampling point.

These results were different depending on the hormonal (GnRH or sexual steroids) milieu of the cells (Fig. 2). Thus, kiss2-12 had a synergic effect with GnRH on LH release. In the presence of sexual steroids (estradiol and testosterone) the stimulatory effect of kiss2-12 on LH and FSH release was reduced.

In the present study, we report the direct action of kiss2 on pituitary gonadotrophs, and demonstrate that this peptide, but not kiss1, elicits modest but significant stimulatory effects on LH and FSH secretion in cultured pituitary cells from spermiating male sea bass. The direct stimulatory effect of kisspeptin on LH release

from gonadotrophs is lower than that of GnRH, which agrees with the accepted notion that the main stimulatory action of kisspeptin on gonadotropin release is mediated via a hypothalamic action. The presence of kiss2 fibres in the sea bass pituitary has been documented (2), suggesting that hypothalamic kiss2 could operate as a hypophysiotropic neuropeptide influencing the function of the gonadotrophs. On the other hand, the *kiss2* gene is expressed in the pituitary of mature male sea bass (3), which also expresses high levels of the receptor genes *gpr54-1b* and *gpr54-2b*. The presence of this ligand/receptor system enables the establishment of an autocrine/paracrine loop in the pituitary. Kiss2 stimulation of LH release was additive to GnRH action, suggesting the possibility that the pathways mediating LH release in response to GnRH and kiss2 in the pituitary may be partially independent. In addition, our results demonstrate that sex steroids modulated the impact of kiss2 on the pituitary. Specifically, testosterone and estradiol reduced the stimulatory action of kiss2 on gonadotropin release.

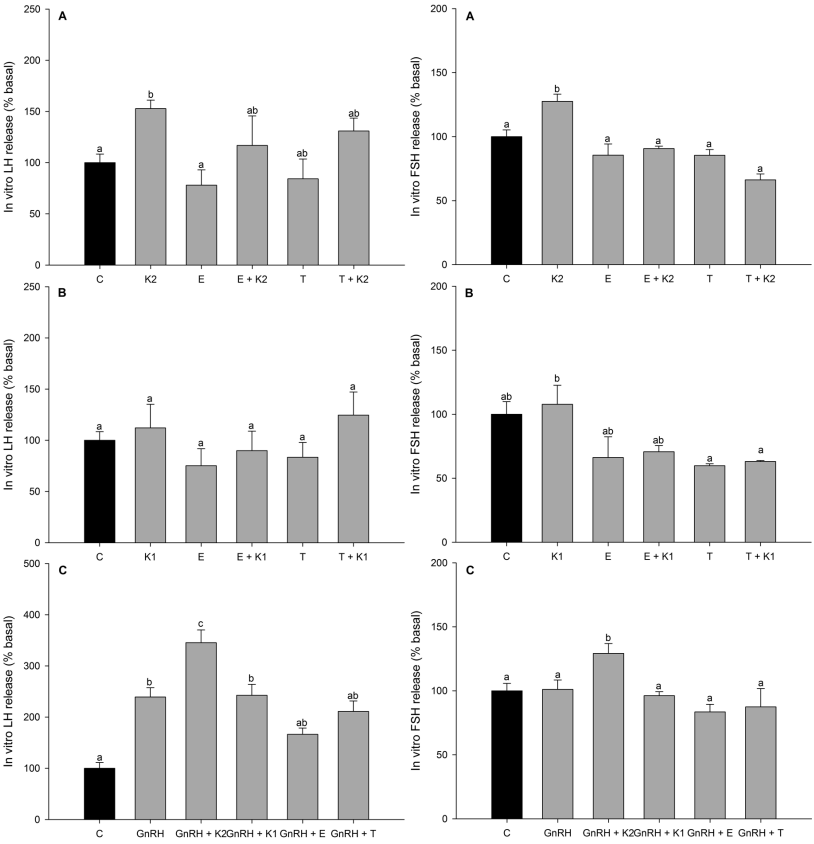

Figure 2. Effects of kiss2-12 (A), kiss1-15 (B) and GnRH (C) in combination with sexual steroids (estradiol and testosterone) on LH release after 8 h of incubation (left) and FSH release after 4 h of incubation (right). Results are expressed as percentage increase over basal secretion. Significant differences between groups are represented by different letters above the bars for each sampling point.

Acknowledgements

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PRODUCTION OF RECOMBINANT EUROPEAN SEA BASS ANTI-MULLERIAN HORMONE PROTEINS USING TWO DIFFERENT EXPRESSION SYSTEMS

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Anti-Müllerian hormone (Amh), also called Müllerian-inhibiting substance, is a member of the TGF-β family. In mammals, Amh is processed to yield a bioactive C-terminal homodimer required for Müllerian duct regression in fetal males. Teleost fish lack Müllerian ducts, but Amh has been identified in several species including the European sea bass. Amh acts by binding and activating a membrane receptor named Amh type II receptor (Amh-RII), which has been described in only four fish species, including sea bass. Here, we report on the production of recombinant sea bass Amh (sbAmh) in two expression systems: *Pichia pastoris* and CHO cells. Using the CHO cells we have produced a recombinant form of the sbAmh that once proteolytically treated with plasmin generates a mature protein. Using the yeast *P. pastoris* expression system we have produced recombinant sbAmh proteins that are endogenously cleaved by the Kex2p enzyme and secreted into the media. Amh was detected by immunohistochemistry in Sertoli cells surrounding early germ-cell generations. During spermatogenesis, staining in Sertoli cells surrounding spermatocytes is weaker. Confirmation of this data came from quantitative PCR experiments showing higher expression levels of *amh* and *amhr2* during early and final stages of spermatogenesis. In conclusion, using two different expression systems we have produced recombinant sbAmh that will be used to study how this growth factor regulates sea bass gametogenesis and steroid release in tissue culture.

Introduction

Anti-Müllerian hormone (Amh) is a dimeric glycoprotein member of the TGF-β family (1). In mammals, Amh is required for Müllerian duct regression in fetal males. Amh is also produced during postnatal life in both male and female regulating steroidogenesis and early stages of folliculogenesis. Amh signals through a transmembrane Amh type II receptor (Amhr2) with serine-threonine kinase activity, which recruits and phosphorylates one or more of the activin-receptor kinases (ALK), mediating stimulatory (ALK2 and 3) or inhibitory (ALK6) effects and involving Smad proteins in the downstream signaling cascade (2).

Teleost fish lack Müllerian ducts, but the *amh* gene has been identified in several species including European sea bass (*Dicentrarchus labrax*). However, *amhr2* has been reported only in medaka, fugu and black porgy. Here, we report on the production of recombinant sea bass Amh (sbAmh) in two expression systems: mammalian CHO cells and *Pichia pastoris*.

Materials and Methods

1. CHO cell expression system. Sea bass *amh* cDNA (AM232701) was cloned into the pcDNA3 expression vector (Invitrogen). The putative cleavage site was optimized changing the RATR-motif to a RARR-motif. A six-His tag was introduced before Ala 430 to facilitate the purification of the mature peptide. The generated plasmid pcDNA3his-amh was used to transfect CHO cells. His-tagged sbAmh expressing cells were selected using the G418 antibiotic.

2. *Pichia pastoris* expression system. Sea bass *amh* cDNA, excluding the signal peptide, was cloned in frame with the α-factor signal sequence into the pPIC9K vector (Invitrogen). The RATR-motif was changed to an EKR site for cleavage of the pro-protein by *P. pastoris* Kex2 protease. A six-His tag was introduced before (pPICK9his-amh) or after (pPICK9amh-his) the mature protein to facilitate purification. These plasmid constructs were used to transform *P. pastoris*. His^+ transformants, resistant to G418 and with multiple copies of transgenes (Mut^+) were selected and grown in 2L flasks with methanol added to the medium as inductor.

3. An antibody directed to sea bass Amh. A rabbit antiserum was produced against the amino acids 430-ADPNNPVRGHTC-441 located immediately downstream of the predicted proteolytic cleavage site (amino acids 426-429). This antibody called anti-C amh, recognizes 12 amino acids immediately after the end of the His-tag.

Results and Discussion

1. Recombinant sbAmh and its cleavage product. CHO stable clones that were resistant to G418 were expanded and tested for the presence of recombinant his-Amh, in both cells and media, by RT-PCR, western blot and immunocytochemistry. Western-blot analysis of up-concentrated culture medium using an antibody directed against the C-terminal end of sbAmh revealed proteins in the range of 66-79 KDa corresponding to the full length Amh and dimers in the range of 140-160 KDa. These secreted pro-peptides were cleaved *in vitro* with plasmin to generate a His-tag mature protein that was then purified by IMAC. The mature peptide corresponded to the expected size, of 12 KDa. In addition, the observation of an intermediate fragment of 32 KDa could indicate the existence of a second cleavage site. On the other hand, several clones of *P pastoris* were obtained producing high levels of recombinant Amh that contained an His-tag either at the N-terminus or the C-terminus of the mature protein. In this case, no proteolitic treatment was neccessary to obtain the mature protein, as the yeast Kex2 protease cleaved *in vivo* the pro-peptide generating a mature sbAmh of the expected size, which was secreted in the culture medium, as detected by western blot.

2. Immunohistochemistry localization of Amh on sea bass testis sections. In sea bass immature testes, a strong staining for amh was detected in Sertoli cells surrounding type A spermatogonia. The signal was weaker in Sertoli cells contacting spermatocytes in testes that had already entered meiosis. In no case was a signal found in the interstitium.

3. Seasonal expression. Sea bass *amh* and *amhr2* are highly expressed in testes during early stages of spermatogenesis (stages I and II), while levels decreased during full spermatogenesis (stages III and IV) (Fig. 1). The re-increase in fully mature fish (stage V) might reflect the decrease in testicular mRNA content in fully mature testes.

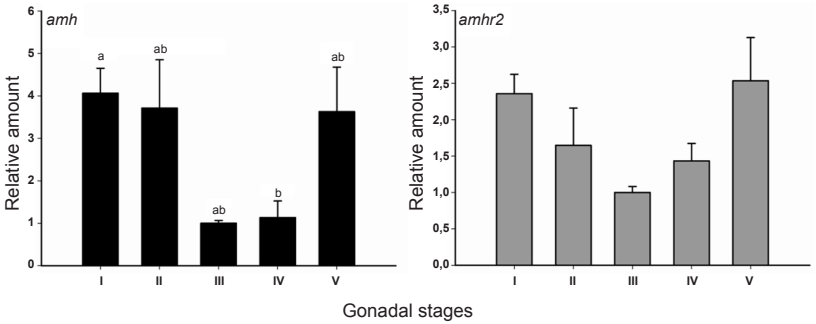

Figure 1. Relative changes in expression of *amh* and *amhr* in sea bass testes sampled during their first sexual maturation. Values, shown as the mean ± SEM, are sorted according to the histological stage of development (3). Stage I (n = 18), immature; stage II (n = 5), early recrudescence; stage III (n = 3), mid recrudescence; stage IV (n = 6), late recrudescence; stage V (n = 8), spermiating testes. Expression data are normalized to *L13a* expression and represented relative to the mean value in stage III. Different letters indicate significant differences between gonadal stages; no significant differences were found as regards *amhr2* mRNA levels.

In conclusion, using mammalian and yeast expression systems we have produced recombinant sbAmh that once exogenously (plasmin) or endogenously (Kex2) processed generate mature proteins, which will be used in functional experiments.

Acknowledgements

Supported by MICINN (AGL2011-28890), EU Project LIFECYCLE (FP7-222719-1) and GV (PROMETEO/2010/003 and ACOMP/2013/085).

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FUNCTIONAL ROLE FOR A CCAAT-BINDING FACTOR IN THE PROXIMAL PROMOTER OF EUROPEAN SEA BASS *KISS2* GENE

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Kisspeptins, encoded by the *Kiss1* gene are key modulators of reproduction in mammals via stimulation of GnRH neurons. In other vertebrate species, including the European sea bass (*Dicentrarchus labrax*), two distinct genes encoding kisspeptins have been described, namely *kiss1* and *kiss2*. In this study, we have isolated 3 Kb of the 5' flanking region of sea bass *kiss2.* The sequence of this fragment has been compared with putative promoter sequences of *kiss2* from several fish, and further analyzed *in silico* for conserved regulatory elements. Based on this analysis, different *kiss2* promoter fragments have been functionally tested for their ability to direct luciferase expression in CHO and HEK 293 cells. Our data indicate that less than 1 Kb promoter is needed to direct maximal luciferase expression in the mentioned cell lines. Further co-transfections of promoter fragments with different predicted transcription factors showed that the CCAAT/enhancer-binding protein alpha and the heterotrimeric transcription factor NFY are able to bind to a conserved proximal CCAATbox, inducing opposite effects on the transcriptional activity of the gene. Although further studies are needed, these results suggest a prominent role of this CCAAT binding sequence in the regulation of *kiss2* expression in sea bass.

Introduction

Kisspeptins have emerged as key modulators of reproduction in mammals via stimulation of GnRH neurons. In mammals, these peptides are encoded by the *KiSS1* gene. In some fish species, including European sea bass (*Dicentrarchus labrax*), two distinct genes encoding kisspeptins exist, *kiss1* and *kiss2*. Studies in sea bass and zebrafish, including *in vivo* kisspeptin administration, *in situ* hybridization and immunohistochemistry, have shown that kiss2 could have a more prominent role than kiss1 in stimulating the gonadotropic axis (1). In addition, knowledge of the transcriptional regulation of these *kiss* genes could also help to understand their roles in fish. In mammals, there are several studies on the promoter of *KiSS1* (2, 3). However, out of a recent study in goldfish *kiss* promoters (4) there is no functional evidence about the transcription factors involved in the regulation of the *kiss* genes in fish. In this study, we have cloned and functionally characterized the putative promoter region of European sea bass *kiss2*.

Materials and Methods

1. Genomic DNA isolation and promoter plasmid constructs. Fragments of genomic DNA located upstream of sea bass *kiss2* cDNA were obtained by genome walking PCR using the Universal GenomeWalker kit (Clontech). *kiss2* promoter segments of different lengths were introduced in the promoterless expression vector pLuc+ that contains the firefly luciferase gene.

2. Bioinformatic tools. *In silico* prediction of transcription factor recognition sites was performed using the DiAlign TF and MatInspector programs from the Genomatix Software server (http://www.genomatix.de).

3. Cell transfection and analysis. Plasmids containing different fragments of *kiss2* promoter upstream of the Luc+ gene were transiently transfected into CHO or HEK293 cells. Transfection efficiency was evaluated by co-expression of a *Renilla reniformis* luciferase plasmid. Luciferase activities were measured with the dual-luciferase reporter assay system (Promega) 24 h after transfection.

Results and Discussion

1. Isolation and *in silico* characterization of the sea bass *kiss2* promoter. 3 kb of putative promoter sequence of sea bass *kiss2* were isolated and sequenced. To look for potential and conserved transcription factor binding sites, 5' upstream sequences of the *kiss2* genes from fugu, stickleback, tetraodon and medaka were extracted from public data bases. Then, these sequences together with the one of sea bass *kiss2* promoter were aligned with DiAlign and potential regulatory sites were analyzed with MatInspector. In this way, a highly conserved sequence was identified in the proximal promoter region and a site for a CAAT-binding factor was predicted.

2. *In vitro* functional characterization of the sea bass *kiss2* promoter. Luciferase plasmids containing 3 Kb, 1 Kb or 178 bp of the sea bass *kiss2* promoter were transiently transfected into CHO or HEK293 cells. The results of luciferase activity obtained showed that the sea bass *kiss2* promoter is active in both CHO and HEK293 cell lines. In CHO cells, the 3Kb fragment was significantly less active than the 1 Kb and 178 bp fragments. This last fragment contains the conserved sequence identified by *in silico* analysis.

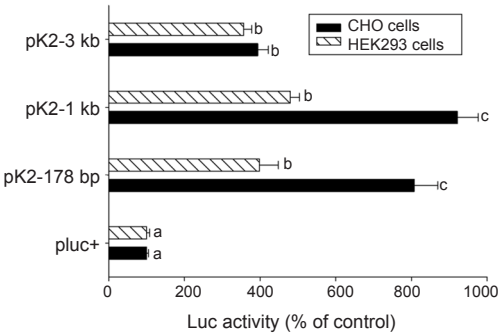

Figure 1. Transcriptional activity of three *kiss2* promoter constructs using HEK293 and CHO cells, and a luciferase reporter system. Luciferase activity was calculated as a quotient between relative light units (RLU): RLU LUC firefly / RLU LUC Renilla, and is shown as a percentage of the activity displayed by the empty vector pLuc+. Bars are means and vertical lines represent SD. Statistical analysis was performed by one way Anova and the Holm-Sidak method for multiple pairwise comparisons.

To test the functionality of this predicted sequence, different plasmids containing CCAAT-binding factors were co-transfected with the kiss2-promoter constructs. We obtained activity with two of these factors. A mouse NFY stimulates *kiss2*

transcription in CHO cells, while zebrafish CEBPα inhibited *kiss2* transcriptional activity. These effects were still obtained when only 178 bp of proximal promoter were used, suggesting that the predicted sequence was the binding site for the tested factors. When different concentrations of these transcription factors were used to co-transfect CHO cells with the 178 bp promoter construct, we obtained a dose-response effect. To further confirm the binding site for the CCAAT-elements site-directed mutagenesis was performed to interrupt the predicted CAAT binding sequence. Three different groups of mutations were introduced into the 1 Kb promoter plasmid, and each construct was transfected into CHO cells with or without CEBPα or NFY. We observed that promoter activity on luciferase expression was totally eliminated when any of the mutations disrupting the conserved binding site were present, independently of the presence of any of the transcription factors.

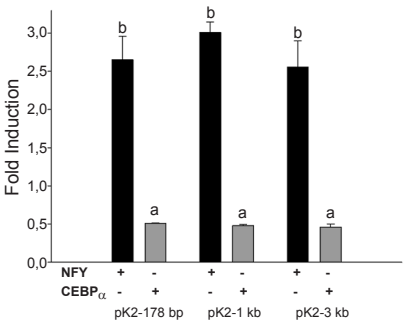

Figure 2. Functional testing of transcription factor binding sites predicted by bioinformatic tools. The promoter-luc plasmids were co-transfected into CHO cells with vectors containing the NFY or CEBPα transcription factors. Co-transfection with empty pcDNA3 was used as control and set as 1 (not shown). Luciferase activity was calculated as in Fig. 1. Bars are means and vertical lines represent SD.

All together, our data indicate that a fragment of 178 bp upstream from the transcription start site of the sea bass *kiss2* gene is able to direct luciferase expression in CHO cells. In addition, a conserved sequence contained in this fragment is essential for promoter activity. Further co-transfections of promoter fragments with the CEBPα or NFY transcription factors revealed that CEBPα downregulates and NFY upregulates *kiss2* promoter activity. Although further studies should be performed, these results suggest a prominent role of a proximal CCAAT binding sequence in the regulation of *kiss2* expression in sea bass.

Acknowledgements

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